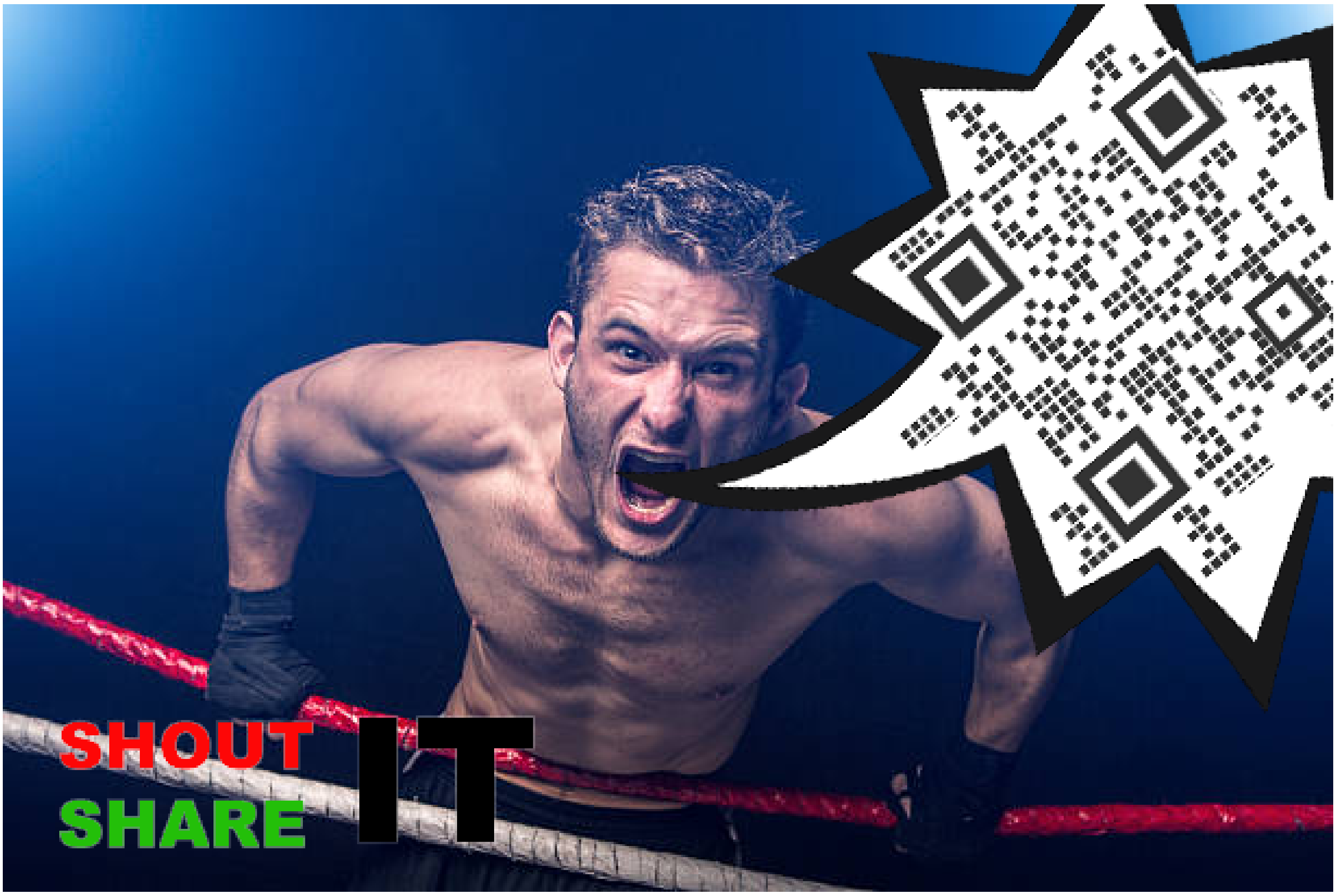
SHOUT
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BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

Editorial Staff

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E. G. Conklin—*Princeton University.*
M. H. Jacobs—*University of Pennsylvania.*
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T. H. Morgan—*Columbia University.*
W. M. Wheeler—*Harvard University.*
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Volume LVII.

WOODS HOLE, MASS.

JULY TO DECEMBER, 1929

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BIOLOGICAL BULLETIN

THE MARINE BIOLOGICAL LABORATORY.

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FORTY-FIRST YEAR.

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I. TRUSTEES.

EX OFFICIO.

FRANK R. LILLIE, *President of the Corporation,* The University of Chicago.
MERKEL H. JACOBS, *Director,* University of Pennsylvania.
LAWRASON RIGGS, JR., *Treasurer,* 25 Broad Street, New York City.
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EMERITUS.

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TO SERVE UNTIL 1932.

H. H. Donaldson, Wistar Institute of Anatomy and Biology.
W. E. Garrey, Vanderbilt University Medical School.
Caswell Grave, Washington University.
M. J. Greenman, Wistar Institute of Anatomy and Biology.
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C. R. Stockard, Cornell University Medical College.

TO SERVE UNTIL 1931.

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B. M. Duggar, University of Wisconsin.
George T. Moore, Missouri Botanical Garden, St. Louis.
W. J. V. Osterhout, Member of the Rockefeller Institute for Medical Research.
J. R. Schramm, University of Pennsylvania.
William M. Wheeler, Bussey Institution, Harvard University.
Lorande L. Woodruff, Yale University.

TO SERVE UNTIL 1930.

E. G. Conklin, Princeton University.
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Ross G. Harrison, Yale University.
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TO SERVE UNTIL 1929.

C. R. Crane, New York City.
I. F. Lewis, University of Virginia.
R. S. Lillie, The University of Chicago.
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Frank R. Lillie, *Ex Off. Chairman.*
Merkel H. Jacobs, *Ex. Off.*
Lawrason Riggs, Jr., *Ex. Off.*
E. G. Conklin, to serve until 1929.
C. R. Stockard, to serve until 1929.
W. E. Garrey, to serve until 1930.
I. F. Lewis, to serve until 1930.

The Library Committee.

C. E. McClung, *Chairman.*
Robert A. Budington.
B. M. Duggar.
E. E. Just.
Frank R. Lillie.
M. M. Metcalf.
Alfred C. Redfield.
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L. L. Woodruff.

MARINE BIOLOGICAL LABORATORY
LIBRARY
WOODS HOLE, MASS.

II. ACT OF INCORPORATION.

No. 3170

Commonwealth of Massachusetts.

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, Henry B. Pierce, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand, Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY.

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 12 o'clock noon, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk, who shall be, *ex officio*, members of the Board of Trustees, and Trustees as hereinafter provided. At the annual meeting to be held in 1897, not more than twenty-four Trustees shall be chosen, who shall be divided into four classes, to serve one, two, three and four years, respectively, and thereafter not more than eight Trustees shall be chosen annually for the term of four years. These officers shall hold their respective offices until others are chosen and qualified in their stead. The President of the Corporation, the Director and the Associate Director of the Laboratory, shall also be Trustees, *ex officio*.

II. Special meetings of the members may be called by the Trustees to be held in Boston or in Woods Hole at such time and place as may be designated.

III. The Clerk shall give notice of meetings of the members by publication in some daily newspaper published in Boston at least fifteen days before such meeting, and in case of a special meeting the notice shall state the purpose for which it is called.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President of the Corporation who shall also be Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The accounts of the Treasurer shall be audited annually by a certified public accountant.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

IX. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. THE REPORT OF THE TREASURER.

To the Trustees of the Marine Biological Laboratory:

Gentlemen: As Treasurer of the Marine Biological Laboratory I herewith submit my report for the year 1928.

The books have been audited by Messrs. Seamans, Stetson & Tuttle. A copy of their report is on file at the Laboratory and is open to inspection by Members of the Corporation.

The book value of the Endowment Fund in the hands of the Central Union Trust Company of New York remains at $906,337.50 in securities and $112.00 in cash. The only changes in investments during the year having been the collection of and reinvestment in, mortgages.

At the end of the year the Lucretia Crocker Fund consisted of securities of the book value of $3,991.09, and cash of $795.42.

The Ida H. Hyde Fund is invested in a mortgage participation of $2,000.

During the year a fund of $2,000 was presented to the Laboratory to found the Bio Club Scholarship of the College of the City of New York and this was also invested in a mortgage participation.

During the year a fund of $3,000 was received by the Laboratory from Mrs. Reynold A. Spaeth to found the Reynold A. Spaeth Memorial Lecture. This fund was also invested in a mortgage participation.

The Retirement Fund at the end of the year consisted of $10,000 invested in mortgage participations, and cash of $76.91.

The land, buildings, equipment and library excluding Devils Lane and Gansett property, represents an investment of $1,575,794.17, less depreciation $171,767.80, or a net amount of $1,404,026.37.

In addition to the special funds above mentioned there were received during the year the following donations:

From General Education Board for improving the facilities of the library	$15,000.00
From Dr. Frank R. Lillie for grading and planting	490.00
Through Dr. Dalgren the sum of	603.62

During the year the Drew mortgage of $10,000 was paid off as well as $1,000 on account of the mortgages on the Danchakoff property, and the indebtedness of the Laboratory at the end of the year consisted of $31,500 in mortgages and $2,315.30 in accounts payable.

Current income for the year was exceeded by expenditures including depreciation by $2,715.71. Over $25,000 was expended from current funds on buildings, equipment, library and reduction in mortgages.

Following is the balance sheet at the end of the year and the condensed statement of income and outgo for the year also the surplus account:

EXHIBIT A.

Marine Biological Laboratory Balance Sheet,

December 31, 1928.

Assets.

Endowment Assets and Equities:		
Securities and Cash in Hands of Trustee—Schedule I	$ 906,449.50	
Securities and Cash—Minor Funds—Schedule II	11,778.04	$ 918,227.54

Plant Assets:			
Land—Schedule IV	$ 113,603.05		
Buildings—Schedule IV	1,205,147.83		
Equipment—Schedule IV	144,319.38		
Library—Schedule IV	112,723.91	$1,575,794.17	
Less Reserve for Depreciation		171,767.80	
		$1,404,026.37	
Cash in Dormitory Building Fund		1,424.21	$1,405,450.58
Current Assets:			
Cash		$ 7,848.51	
Accounts—Receivable		19,078.17	
Inventories:			
Supply Department	$ 30,418.67		
Biological Bulletin	6,867.68	$ 37,286.35	
Investments:			
Devil's Lane Property	$ 34,743.31		
Gansett Property	1,942.39		
Stock in General Biological Supply House, Inc.	12,700.00		
Retirement Fund Assets	10,076.91	$ 59,462.61	
Prepaid Insurance		4,268.49	
Items in Suspense		8.40	$ 127,952.53

Liabilities.

Endowment Funds:		
General Endowment Funds—Schedule III	$ 906,449.50	
Minor Endowment Funds—Schedule III	11,778.04	$ 918,227.54
Plant Funds:		
Donations and Gifts—Schedule III	$1,011,233.09	
Other Investments in Plant from Gifts and Current Funds	387,717.49	
	$1,398,950.58	
Mortgage, Danchakoff Estate	6,500.00	$1,405,450.58
Current Liabilities and Surplus:		
Mortgage, Devil's Lane Property	$ 25,000.00	
Accounts—Payable	2,315.30	
	$ 27,315.30	
Current Surplus—Exhibit C	100,637.23	$ 127,952.53

MARINE BIOLOGICAL LABORATORY
LIBRARY
WOODS HOLE, MASS.

EXHIBIT B.

Marine Biological Laboratory Income and Expense,

Year Ended December 31, 1928.

	Total.		Net.	
	Expense.	Income.	Expense.	Income
Income, Endowment Fund ..		$ 46,702.04		$ 46,702.04
Donations (See also Current Surplus)		603.62		603.62
Instruction	7,462.26	10,005.00		2,542.74
Research	4,028.40	16,550.00		12,521.60
Biological Bulletin and Membership Dues	6,974.83	8,183.88		1,209.05
Supply Department, Schedule V....................	57,997.30	68,627.52		10,630.22
Mess, Schedule VI.........	32,644.52	35,361.86		2,717.34
Dormitories, Schedule VII..	31,591.74	13,378.45	18,213.29	
(Interest and Depreciation charged to above three Depts. See Schedules V., VI., and VII.)	*35,313.05*			35,313.05
Dividends on Stock, General Biological Supply House, Inc.		2,032.00		2,032.00
Rent, Danchakoff Cottages .	263.43	350.00		86.57
Rent, Microscopes		400.00		400.00
Rent, Garage, Railway, Etc.		256.10		256.10
Rent, Newman Cottage	81.47	150.00		68.53
Interest on Bank Balances ..		136.36		136.36
Medical Fees		59.00		59.00
Sundry Items		29.26		29.26
Maintenance of Plant:				
New Laboratory Expense	16,128.73		16,128.73	
Maintenance, Buildings and Grounds	10,075.64		10,075.64	
Chemical and Special Apparatus	8,503.41		8,503.41	
Library Dept. Expenses ..	8,897.45		8,897.45	
Carpenter Dept. Expenses	2,256.67		2,256.67	
Truck Expenses	1,014.62		1,014.62	
Sundry Expenses	460.90		460.90	
Bar Neck Property Expense	354.00		354.00	
Evening Lectures	137.01		137.01	
Workmen's Compensation Insurance	869.70		869.70	
General Expenses:				
Administration Expenses .	13,247.49		13,247.49	
Interest on Loans	734.33		734.33	

Endowment Fund Trustee	787.50		787.50	
Bad Debts	505.83		505.83	
Contribution for Research space, Naples Zoological Station	250.00		250.00	
Reserve for Depreciation	35,586.62		35,586.62	
Excess of Expense over Income carried to Current Surplus—Exhibit C		2,715.71		2,715.71
	$205,540.80	$205,540.80	$118,023.19	$118,023.19

EXHIBIT C.

MARINE BIOLOGICAL LABORATORY, CURRENT SURPLUS ACCOUNT, YEAR ENDED DECEMBER 31, 1928.

Balance, January 1, 1928		$ 91,700.01
Add:		
Donations Received,		
From General Education Board for purchase of Books for Library		15,000.00
From Dr. Frank R. Lillie for Grading, Planting, etc., on Laboratory Grounds		490.00
Income from Retirement Fund		253.80
Reserve for Depreciation charged to Plant Fund		35,586.62
		$143.030.43
Deduct:		
Payments from Current Funds during Year for Plant assets as shown in Schedule IV.,		
Buildings	$ 1,351.28	
Equipment	4,788.39	
Library Books, etc.	8,106.06	
	$14,245.73	
Payments from above Donations charged to Plant Assets,		
General Education Board, Purchase of Books	13,934.98	
Dr. Frank R. Lillie, Grading, etc.	490.00	
Payments on Mortgages,		
Drew Estate	10,000.00	
Danchakoff Estate	1,000.00	
Adjustment Supply Department Accounts—Receivable	6.78	
Balance of Income-and-Expense Account—Exhibit B	2,715.71	42,393.20
Balance, December 31, 1928—Exhibit A		$100,637.23

Respectfully submitted,

LAWRASON RIGGS, JR.,

Treasurer.

V. THE REPORT OF THE LIBRARIAN, DECEMBER 31, 1928.

The Library contains 26,527 bound volumes; 22,000 of these, or about five-sixths, are in the form of scientific journals. The other one-sixth, or 4,527, are books of reference. There are 51,000 reprints filed and catalogued under the authors' names. The following comparative figures extending through the past five years are both interesting and gratifying. In 1923, there were in the Library 11,698 bound volumes (books and serials); in 1924, 1,302 were acquired; in 1925, 2,000; in 1926, 3,200; in 1927, 4,542; in 1928, 3,765; a total increase in these years of 14,809, or the original number was more than doubled. In 1923, there were in the library 9,587 reprints; in 1924, 213 were added; in 1925, 15,200; in 1926, 13,000; in 1927, 5,000; in 1928, 8,000; a total addition of between four and five times the number that there were in 1923.

The serial list comprises 1,421 titles. Of these only 874 are received currently. During the year, 302 of these were paid from the budget at a cost of $4,220.93. The others were received by exchange, about 350, and by gift, about 222. Properly the cost of exchanges should appear in a reckoning of any total cost. At least 273 annual subscriptions of the Biological Bulletin at $8.10 each were sent out for these so that about $2,200 additional should appear on this item of expenditure. When $1,200 is added for the cost of binding, the annual outlay for the current serials mounts to a total of $7,620.93. The number of serials exceeds the list of 1927 current titles by 74. Thirty-five (35) of these new serial publications came by subscription, 31 by exchange, and 8 by gift. The back sets of serials from the General Education Board fund reach (in round numbers) 1,270 volumes for 1928, at an expenditure of $6,762.24 plus a sum of $2,191.33 on binding (estimated total per volume, $7.00); the Comptes Rendus de la Société de Biologie being the most valuable set secured. In the past three years, 3,714 serial volumes have been purchased and $26,280.42 spent on these, besides $3,613.72 expended on binding. About $1,000 of this last sum, however, was for back sets secured on exchange and should not be reckoned with the total cost of the above 3,714 volumes.

Of the 26,527 volumes in the Library, only 4,527 are classed as books. But the proportion of books compared with the serial volumes has steadily gained in spite of the expressed policy to give precedence always in this Library to the original publications contained in journals. In 1925, the proportion was 1 to 9; in 1926, 1 to 8; and in 1928, 1 to 6. This increase is in large part accountable to the gifts of books, both new and old, and to the purchase of the library on Crustacea of Sidney I. Smith. This year also an unusual number of new currently issued books were purchased on the regular budget of the Library, 238, a figure never before reached in one year. At the same time, purchase in the past three years of many important sets of monographs and bound volumes of expeditions and other systematic material, as well as handbooks in physics, chemistry and biology, has expanded our book holdings and has vastly increased the reference facilities. Seven hundred and forty-six (746) such monographs is the total number secured through the General Education Board to date, 447 of these having been secured in 1928 besides 241 volumes of the above mentioned library on Crustacea. The cost of the total 987 volumes (books) was $6,814.69 (estimated cost per volume, $7.00). Saccardo's "Sylloge Fungorum" is the most notable reference work so far purchased, but the "Valdivia Expedition," Abderhalden's "Arbeitsmethoden" and Geiger's "Handbuch der Physik" were very expensive also. The sum paid for the Sidney I. Smith library has been charged only against books, whereas 1,516 reprints and many volumes of serials were secured in this huge library. But the library was purchased chiefly for the reference books on Crustacea, and the reprints though forming a very important part are difficult to evaluate and the serial numbers and volumes though filling in 107 gaps, were distributed among nearly as many sets. The entire sum therefore has been charged against books.

During the year, 3,000 reprints of current issue were received by the Library and were promptly catalogued and filed by author. No subject cards of these were made. There were 5,000 reprints filed that had been received in the Whitman and other libraries and also 6,878 not new in our possession but heretofore inadequately catalogued. Only those reprints falling in date between 1912 and

1925 were catalogued under subjects. Since all general notices and sporadic personal appeals heretofore attempted to secure complete sets of reprints of the investigators working at the Laboratory, had failed, a canvas was made of the persons working here and the question of filling in for the Library his complete set of reprints and of supplying a bibliography of his published work was put directly to each. By letters following up this personal appeal, the names are being gradually checked as sets are secured to date and current issued are promised.

The total expenditures of the Library were as follows: books, $526.82; serials, $4,220.93; binding current serials, $1,093.78; binding back sets, $2,191.33; express, $213.80; supplies, $534.48; salaries, $8,000; back serial sets, $6,762.24; monographs and handbooks, $4,822.67; and a sum of $2,200 for about 273 sets of the BIOLOGICAL BULLETINS used in exchanges. The total sum administered in connection with the Library was therefore $30,566.05. Besides this, the sum of $44.10 was placed to our credit through the sale of duplicate material. Of this total as given above, $8,000 or 26 per cent. (or scarcely more than one quarter of the whole) was used in administration or in salaries constituting (along with postage and express) the Library's only overhead. A brief analysis of the work covered by the salary item of expenditure and a segregation of these items is of interest. During the year, as has been already stated, 14,878 reprints and 3,765 bound volumes were shelved. The cost of shelving the 14,878 reprints at $.16 each was $2,380.48. (In 1929, this will be reduced to $.15 each.) The cost in time of securing these reprints has until this past summer been practically nil. A money value for the time so spent this summer can be included with the time spent in the circulation of the library material in the summer, and a round sum to cover the value of this time can be set at $1,200. It may be parenthesized here that the count of charge cards is 2,209 for 1928, 363 more than for 1927, in spite of the fact that serials were not allowed to be charged out. Also 28 mailing loans were handled by the Secretary and 113 volumes were borrowed. With $3,580.48 allowed on reprints and circulation, the remaining sum of $4,420.52 was used in personnel to secure and to shelve (including cataloguing) the 3,765 volumes acquired this year. This shows the average cost per volume for serials and books to have been $1.18 in contrast to $.16 ($.15 in the future) for each reprint. The time element in getting the books and serials will, if properly pursued, increase in the future rather than lessen because many of the volumes now lacking are increasingly harder to find. The same point holds in regard to securing exchanges. And, for the reprints, effort must be made to secure authors' complete sets if they are to retain in the future the value, entirely independent of the serials, that has so far characterized their use. As time goes on there will be an increasing number of reprints that must be bound and the expense of such binding should be separately listed. Occasion often arises when reprints must be purchased. A sum of money to be spent in securing and in binding reprints will be recommended, therefore, by the Librarian on the budget for 1930.

There were presented to the Library by the authors and the publishers books (and publications) as follows. Grateful acknowledgment and cordial thanks is hereby expressed on the part of the Library for these gifts.

From the publisher, Baird & Tatlock Ltd.: *Standard Catalogue of Scientific Apparatus, Vol. I.—Chemistry.*

P. Blakiston's Son & Co.: Craigie, E. Horne: *An Introduction to the Finer Anatomy of the Central Nervous System based upon that of the Albino Rat*; Evans, C. Lovatt: *Recent Advances in Physiology*; Gould, George M.: *Medical Dictionary*; Hawk, Philip B.: *Practical Physiological Chemistry*; Kingsley, J. S.: *Outlines of Comparative Anatomy of Vertebrates*; Kostychev, S.: *Plant Respiration*; Lee, Arthur Bolles: *Microtomist's Vade-mecum*; Webster, R. W.: *Diagnostic Methods*; and Woollard, H. H.: *Recent Advances in Anatomy.*

The Century Co.: Hegner, Robert: *Host-parasite Relations between Man and his Intestinal Protozoa*; and Yerkes, Robert M.: *Almost Human.*

Chemical Catalog Co.: Palmer, Leroy S.: *Carotinoids and Related Pigments.*

Chicago University Press: Allee, W. C.: *Synoptic Key to the Phyla, Classes, and Orders of Animals*; Bensley, Robt. Russell: *The Structure of the Glands of Brunner*; Jordan, Edwin O. and Falk, I. S.: *The Newer Knowledge of Bacteriology and Im-*

munology; Lillie, F. R. and Moore, C. R.: *A Laboratory Outline of Embryology, Chick and Pig*; McNair, James B.: *Rhus dermatitis (Poison Ivy), Its Pathology and Chemotherapy*; Shambaugh, Geo. E.: *Blood-vessels in the Labyrinth of the Ear*; and Tower, Wm. L.: *Colors and Color Patterns of Coleoptera.*

The Fleischmann Laboratories: Sorensen, S. P. L.: *Proteins.*

L. Friederichsen & Co.: Michaelsen, W.: *Beiträge zur Kenntnis der Land- und Süsswasserfauna Deutsch-Südwestafrikas, Vol. II, Lief. 4–5 and Schlussband.*

Harvard University Press: McAdie, Alex.: *Man and Weather*; and Wetmore, Alex.: *The Migrations of Birds.*

Paul B. Hoeber, Inc.: Cowdry, Edmund V., Editor: *Special Cytology.*

Henry Holt & Co.: Burlingame, Heath, Martin and Peirce: *General Biology.*

Houghton Mifflin Co.: Ellis, Havelock: *A Study of British Genius.*

Alfred A. Knopf Co.: Cumston, C. G.: *An Introduction to the History of Medicine*; and de Réaumur, René Antoine Ferchault: *The Natural History of Ants*; and Pearl, Raymond: *Alcohol and Longevity.*

Lea & Febiger: Kilduffe, Robert A.: *The Clinical Interpretation of Blood Chemistry*; Martin, Ernest G. and Weymouth, F. W.: *Elements of Physiology for Students of Medicine and Advanced Biology.*

Little, Brown, & Co.: Boyle, Mary E.: *In Search of Our Ancestors.*

Longmans, Green & Co.: Sharpey-Schafer, Edward: *Experimental Physiology.*

McGraw-Hill Book Co.: Babcock, E. B. and Clausen, R. E.: *Genetics in Relation to Agriculture*; Daniels, Farrington: *Mathematical Preparation for Physical Chemistry*; Eucken, Arnold: *Fundamentals of Physical Chemistry*; Gavett, George Irving: *First Course in Statistical Method*; Hill, A. V.: *Muscular Movement in Man*; Loeb, Leonard B.: *Kinetic Theory of Gases*; Schorger, A. W.: *Chemistry of Cellulose and Wood*; and Weiser, H. B.: *Hydrous Oxides.*

Macmillan Co.: Bruner, H. L.: *Laboratory Directions in Col-*

lege Zoölogy; Clark, A. I.: *Comparative Physiology of the Heart*; Gray, James: *Ciliary Movement*; Harvey, H. W.: *The Biological Chemistry and Physics of Sea Water*; Hogben, L. T.: *The Comparative Physiology of Internal Secretion*; Johnson, M. E., and Snook, H. J.: *Seashore Animals of the Pacific Coast*; Little, M. E., and Kempton, R. T.: *A Laboratory Manual for Comparative Anatomy*; Mason, F., Editor: *Creation by Evolution*; Parker, T. J. and Haswell, W. A.: *Text-book of Zoölogy*; Shumway, Waldo: *The Frog*; and Warbasse, James P.: *Coöperative Democracy.*

William Morrow & Co.: Stanford, Alfred: *The Navigator, the Story of Nathaniel Bowditch.*

New York University Press Book Store: Smith, Bertram G.: *Laboratory Guide in Microscopical Anatomy.*

The Open Court Publishing Co.: Calkins, Mary Whiton: *The Metaphysical System of Hobbes*; Hering, Ewald: *Memory*; Kant: *Prolegomena*; Lao-tze: *The Canon of Reason and Virtue*; Leibniz: *Discourse of Metaphysics*; Mach, Ernst: *The Analysis of Sensations and the Relation of the Physical to the Psychical*; Reitz, H. L.: *Mathematical Statistics*; Rignano, Eugenio: *Essays in Scientific Synthesis*; Russell, Bertrand: *Our Knowledge of the External World*; St. Anselm: *Proslogium Monologium*; and Stillman, John M.: *Päracelsus.*

W. B. Saunders Co.: Burton-Opitz, Russell: *A Text-book of Physiology for Students and Practitioners of Medicine*; Drew, Gilman A.: *A Laboratory Manual of Invertebrate Zoölogy*; Howell, W. H.: *Text-book of Physiology for Medical Students and Physicians*; McLester, James S.: *Nutrition and Diet in Health and Disease*; Palfrey, F. W.: *The Specialties in General Practice*; Ranson, S. W.: *The Anatomy of the Nervous System*; Rivas, Damaso: *Human Parasitology with Notes on Bacteriology, Mycology, etc.*; and Todd, J. C.: *Clinical Diagnosis by Laboratory Methods.*

G. E. Stechert & Co. (Alfred Hafner): Sobotta-McMurrich: *Atlas of Human Anatomy.*

Toronto University Press: Taylor, Wilson: *A New View of Surface Forces.*

Fred. Warne & Co.: Russell, F. S. and Yonge, C. M.: *The Seas.*

W. Watson & Sons: *Catalogue of Optical and Scientific Instruments and Dictionary.*

John Wiley & Sons: Curtis, W. C. and Guthrie, M. J.: *Textbook of General Zoölogy*; Holman, Richard M. and Robbins, W. W.: *A Textbook of General Botany for Colleges and Universities*; Kingsbury, B. F., and Johannsen, O. A.: *Histological Technique*; Morrow, Clarence Austin: *Biochemical Laboratory Methods for Students of the Biological Sciences*; and Owens, Charles Elmer: *Principles of Plant Pathology.*

The following books were presented by the authors: Conklin, E. G.: *Laboratory Directions in General Biology*; Herrick, J. C.: *Mechanism of the Odontophoral Apparatus in Sycotypus Canaliculatus*; Krafft, Carl F.: *Spiral Molecular Structures the Basis of Life*; Osborn, Henry Fairfield: *From the Greeks to Darwin*; *Impressions of Great Naturalists*; *Evolution and Religion in Education*; *Creative Education in School, College, University, and Museum*; Pratt, Henry Sherring: *A Course in General Biology*; Ramaley, Francis: *Colorado Plant Life*; Rice, F. O., Fryling, C. F., and Wesolowski, W. A.: *The Relation between the Temperature Cœfficient and the Mechanism of a Chemical Reaction*; Rice, F. O.: *A Theory of Chemical Reactivity*; Warbasse, James P.: *What is Coöperation?*; Williams, Wm. A.: *The Evolution of Man Scientifically Disproved*; and Wilson, E. B.: *The Cell in Development and Heredity.*

A most notable gift of books, serials, and reprints to the Library was made by Prof. M. M. Metcalf. The reprints that we can use are about 2,000. The books and serials that were not duplicates number 121, and many very important gaps in our biological Library of both books and serials have thus been filled.

Dr. Baldwin Lucké presented an engraving of Prof. Joseph Leidy which forms a valuable addition to the Library's portrait collection.

VI. THE REPORT OF THE DIRECTOR.

TO THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY:

Gentlemen: I beg to submit herewith a report of the forty-first session of the Marine Biological Laboratory for the year 1928.

1. *Attendance.*—An examination of the Tabular View of Attendance on page —— shows no very significant change in the number of students in the courses as compared with previous years. This is due to the fact that numbers are limited by action of the Executive Committee. The number of investigators, however, has continued to increase, being 323 in 1928 as compared with 294, 252, 207 and 194 in the years 1927, 1926, 1925 and 1924 respectively. That the rate of increase during the past year has been somewhat less than that for the two immediately preceding it and that this rate will probably continue to fall off in the future is a necessary consequence of the near approach to the "point of saturation" during the middle of the summer of the present facilities of the Laboratory. The degree of crowding during this period, already discussed in the Director's reports for 1926 and 1927, is so great that every effort is now being made to encourage attendance during the months when there is no lack of space. The attention of our investigators is invited to the facts that during June and September very desirable research rooms are available in the New Laboratory, that apartments and other living accommodations may be rented at rates considerably lower than those prevailing during the middle of the summer and that the period of operation of the Mess has been extended to include almost the whole of the two months in question. It is hoped that the new system of conducting the courses of instruction in two consecutive periods of six weeks each, which will be tried for the first time in 1929, will have a further effect in spreading the attendance and in so relieving the congestion.

That considerable progress has been made during the past year in lengthening the season in both directions is indicated by the following figures for the number of investigators in attendance on selected days, separated by approximately equal intervals, during the years 1927 and 1928. These figures are of further interest in permitting calculations to be made which show that the time spent at the Laboratory by each investigator was on the average approximately 69 days in 1928 as compared with 67 in 1927.

	1927	1928
March 30	0	0
April 10	0	1

April 20	0	4
“ 30	1	4
May 10	3	7
“ 20	6	10
“ 30	7	15
June 10	50	64
“ 20	114	140
“ 30	212	240
July 10	247	281
“ 20	247	282
“ 30	245	272
August 10	234	250
“ 20	208	226
“ 30	168	183
September 10	110	112
“ 20	50	43
“ 30	12	14
October 10	8	9
“ 20	2	4
“ 30	2	4
November 10	0	4
“ 20	0	4
“ 30	0	4
December 10	0	4
“ 20	0	4
“ 30	0	0

2. *The Report of the Treasurer* shows an increase in the total assets of the Laboratory from $2,449,624.06 in 1927 to $2,451.630.65 in 1928. The income for the past year was for the first time in excess of two hundred thousand dollars, the exact sum being $202,825.09, an increase of approximately nine thousand dollars over the corresponding figure of $193,707.42 for the preceding year. Especially noteworthy is the increasing financial support received from institutions and individuals in connection with the rental of research space. This increase is partly due to the larger number of investigators now in attendance as compared with earlier years and partly to the growing ability of institutions to pay for all of the space occupied by their representatives at the current rates. The receipts from this source for the past five years have been:

1924	$ 6,875.00
1925	$ 9,575.00
1926	$11,650.00
1927	$14,525.00
1928	$16,550.00

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In spite of the very gratifying increase in income, other circumstances have operated to prevent the appearance of a favorable balance in a business sense. The larger the holdings of the Laboratory the greater become the amounts that must be charged to depreciation and interest. The completion of the new Dormitory and Apartment House during the past year have added to these charges the sum of $5,545.70 without a corresponding increase in income, with the result that there is for 1928 a deficit on paper of $2,715.71. This may be compared with similar deficits for 1927 and 1926 of $91.06 and $2,203.69, respectively. However, in view of the very great increase in the depreciation and interest charges for 1928 over the corresponding figures for other years the present showing is in reality decidedly encouraging and it gives grounds for the hope that by a somewhat further lengthening of the season of occupancy of the buildings in question the deficit may ultimately be eliminated completely. As there were no major building operations in 1928 it was possible for the Laboratory after making expenditures from current funds of $8,106.06 for books, $4,788.39 for equipment and $1,351.28 for additions and improvements to existing buildings to pay off during the year $11,000.00 of its total indebtedness, in the form of mortgages, of $42,500.00. This reduction will very considerably decrease the future payments of interest and correspondingly improve the financial condition of the Laboratory.

In addition to the contribution of $15,000 to the Library by the General Education Board and the lectureship and scholarship funds noted elsewhere appreciative mention should be made of gifts to the Laboratory of $603.62 through Dr. Ulric Dahlgren from the Philadelphia Association of the Marine Biological Laboratory and of $490.00 from Dr. Frank R. Lillie, the latter sum being an addition to his previous gift of $1500.00 for the purpose of beautifying the grounds of the Laboratory.

3. *The Report of the Librarian* shows a continuation of the

rapid increase in the library facilities of the Laboratory which has been in progress for several years. The bound volumes (books and serials) added during the past year number 3,756 and the reprints approximately 8,000, bringing the totals for these two classes of publications to 26,500 and 51,000 respectively. As the Librarian points out in her report, there has been within a five year period more than a doubling of the number of bound volumes in the Library and a five-fold increase in the number of catalogued reprints. The substantial gains during 1928 were made possible chiefly by a contribution of $15,000.00 from the General Education Board and by the expenditure of approximately $8,000 (including the value of exchanges) from the general funds of the Laboratory. Further additions were due to the generosity of the various publishers and individuals listed in the Librarian's report, of whom Professor M. M. Metcalf should be especially mentioned because of the unusual value of his contribution of books and reprints. During the past year the number of serials currently received by the Library has increased from approximately 800 to 874.

4. *Evening Lectures.*—Among the new problems raised by the greatly increased attendance in recent years has been that of making the evening lectures more representative of the work of the Laboratory as a whole than is possible with a series—originally entirely adequate—of two lectures a week for six weeks. At the rate of twelve lectures a year, approximately 18 years would be required for each of the independent investigators in attendance in 1928 to report upon his work to his colleagues. A further problem has been to restore if possible something of the informality and freedom of discussion that characterized these lectures in the early days of the Laboratory before the audiences had reached their present large size.

To meet the changed conditions the experiment was tried during the summer of 1928 of reducing the more formal lectures to one a week, at the same time considerably lengthening the season, and of adding on one or two evenings of the week scientific meetings at which there could be presented groups of three or four shorter and more or less closely related papers in such fields as, for example, Cytology, Genetics, Cell Physiology, etc. Because of the more specialized nature of these papers it was believed that the attendance at any given meeting would be limited to the group of investigators most interested in the field in question and that conditions would thereby be made less formal and discussion encouraged.

On the whole the results of the experiment have been very promising. During the summer of 1928, in addition to the more formal lectures, 12 special sessions were held and at them 39 of the investigators of the Laboratory, instead of the maximum of 12 possible under the old system, reported upon their work. The attendance at most of the meetings was unexpectedly large, but fortunately the desired spirit of informality was not destroyed and the discussion was not only free at all times, but on several occasions was sufficiently heated to recall to the older investigators memories of the earlier days of the Laboratory. It is encouraging to find that to the obvious advantages of bringing together the present large number of investigators there need not necessarily be added the disadvantage of an undesirable degree of formality, which to many had appeared almost an inevitable result of the growth of the Laboratory. It is planned to continue these meetings in future years with such changes in procedure from time to time as experience shows to be desirable.

5. *The Biological Bulletin.*—At a meeting of the Editorial Board of the Biological Bulletin held early in September and at a subsequent meeting of the Executive Committee it was decided that the relation of the journal to the Marine Biological Laboratory could be made closer and the field of its activities somewhat broadened by increasing the size of the Editorial Board. The following were accordingly elected to the Board and all have indicated their willingness to serve the Laboratory in this capacity: Professors E. N. Harvey, S. Hecht, H. S. Jennings, E. E. Just, G. H. Parker and A. C. Redfield.

6. *Scholarships.*—In 1928 there was added to the funds held by the Laboratory for the support of institutional scholarships one of $2,000.00 raised for this purpose by the Bio Club of the College of the City of New York. It is provided in the agreement between the Laboratory and the Bio Club that the income from this fund shall be used to pay the fees for instruction or research at the Marine Biological Laboratory of one or more advanced stu-

dents or investigators from the institution in question and that in the event that no such student or investigator is chosen the income for the year may in its discretion be used by the Laboratory for its general purposes.

There were also placed at the disposal of the Laboratory for the year 1928 by Mr. Ware Cattell, Editor of the *Collecting Net*, five scholarships of $100.00 each. These scholarships were awarded by a Committee to five students who had worked at the Marine Biological Laboratory during the preceding year and who had impressed their instructors as showing unusual promise as investigators. These scholarships, which are to be renewed in 1929, fill a real need of the Laboratory in making it possible to provide for a number of worthy applicants regardless of their institutional connections.

7. *The Spaeth Memorial Lectureship.*—During the past year the Laboratory accepted a generous gift of $3,000.00 from Mrs. R. A. Spaeth to establish a lectureship in memory of her husband, Dr. Reynold Albrecht Spaeth who for a number of years had been closely associated with the Marine Biological Laboratory both as an investigator and as a member of the staff of the Physiology Course, and whose death in Siam two years ago ended a most promising scientific career. It is very gratifying to Dr. Spaeth's many friends that his memory will be kept alive in a manner which is not only highly appropriate in itself, but which will serve perpetually to keep before the other workers at the Marine Biological Laboratory in the fields in which his interests chiefly lay the high scientific standards of the leaders in these fields who will from time to time be invited to deliver the Spaeth Memorial Lecture.

8. *The Hyatt Memorial Tablet.*—At the request of the Executive Committee, Mrs. Alfred G. (Harriet Hyatt) Mayor, daughter of Alpheus Hyatt, prepared during the past year a *bas-relief* and memorial tablet in bronze of her father, which was unveiled in the reading-room of the library on September 4. Addresses were delivered on this occasion by Professor E. G. Conklin who presented the tablet to the Laboratory on behalf of Mrs. Mayor and her family and by Professor Frank R. Lillie who accepted it for the Laboratory. In addition to the portrait of Professor Hyatt, the tablet bears the following inscription:

ALPHEUS HYATT

FIRST PRESIDENT OF THE WOODS HOLE LABORATORY.

HE ALSO FOUNDED ITS PROTOTYPE AT ANNISQUAM, MASSACHUSETTS; ESTABLISHED IN 1880 WITH THE AID OF THE WOMAN'S EDUCATION ASSOCIATION AND THE BOSTON SOCIETY OF NATURAL HISTORY.

1838–1902

There are appended as parts of this report:

1. The Staff, 1928.
2. Investigators and Students, 1928.
3. A Tabular View of Attendance, 1924–1928.
4. Subscribing and Coöperating Institutions, 1928.
5. Evening Lectures, 1928.
6. Shorter Scientific Papers, 1928.
7. Members of the Corporation, August, 1928.

I. THE STAFF, 1928.

Merkel H. Jacobs, *Director*, Professor of General Physiology University of Pennsylvania.

Associate Director: ——— ———.

I. INVESTIGATION.

Gary N. Calkins, Professor of Protozoölogy, Columbia University.

E. G. Conklin, Professor of Zoölogy, Princeton University.

Caswell Grave, Professor of Zoölogy, Washington University.

H. S. Jennings, Professor of Zoölogy, Johns Hopkins University.

Frank R. Lillie, Professor of Embryology, The University of Chicago.

C. E. McClung, Professor of Zoölogy, University of Pennsylvania.

S. O. Mast, Professor of Zoölogy, Johns Hopkins University.

T. H. Morgan, Professor of Experimental Zoölogy, Columbia University.

G. H. Parker, Professor of Zoölogy, Harvard University.

E. B. Wilson, Professor of Zoölogy, Columbia University.

Lorande L. Woodruff, Professor of Protozoölogy, Yale University.

II. INSTRUCTION.

J. A. Dawson, Instructor in Zoölogy, Harvard University.

Rudolf Bennitt, Associate Professor of Zoölogy, University of Missouri.

E. C. Cole, Associate Professor of Biology, Williams College.

T. H. Bissonnette, Professor of Biology, Trinity College.

Madeleine P. Grant, Assistant Professor of Zoölogy, Mount Holyoke College.

E. A. Martin, Assistant Professor of Zoölogy, College of the City of New York.

A. E. Severinghaus, Instructor in Zoölogy, Columbia University.

Donnell B. Young, Professor of Zoölogy, University of Maine.

PROTOZOÖLOGY.

I. INVESTIGATION.

(*See Zoölogy.*)

II. INSTRUCTION.

Gary N. Calkins, Professor of Protozoölogy, Columbia University.

Mary Stuart MacDougall, Professor of Zoölogy, Agnes Scott College.

W. B. Unger, Assistant Professor of Zoölogy, Dartmouth College.

EMBRYOLOGY.

I. INVESTIGATION.

(*See Zoölogy.*)

II. INSTRUCTION.

Hubert B. Goodrich, Professor of Biology, Wesleyan University.

Benjamin H. Grave, Professor of Biology, Wabash College.

Charles Packard, Assistant Professor of Zoölogy, Institute of Cancer Research, Columbia University.

Harold H. Plough, Professor of Biology, Amherst College.

Charles G. Rogers, Professor of Comparative Physiology, Oberlin College.

PHYSIOLOGY.

I. INVESTIGATION.

Harold C. Bradley, Professor of Physiological Chemistry, University of Wisconsin.

Walter E. Garrey, Professor of Physiology, Vanderbilt University Medical School.

Ralph S. Lillie, Professor of General Physiology, The University of Chicago.

Albert P. Mathews, Professor of Biochemistry, The University of Cincinnati.

II. INSTRUCTION.

Merkel H. Jacobs, Professor of General Physiology, University of Pennsylvania.

Edwin J. Cohn, Assistant Professor of Physical Chemistry, Harvard University.

Wallace O. Fenn, Professor of Physiology, University of Rochester.

E. Newton Harvey, Professor of Physiology, Princeton University.

Charlotte Haywood, Assistant Professor of Physiology, Vassar College.

Selig Hecht, Associate Professor of Biophysics, Columbia University.

Leonor Michaelis, Resident Lecturer in Medical Research, Johns Hopkins University.

Alfred C. Redfield, Assistant Professor of Physiology, Harvard University.

BOTANY.

I. INVESTIGATION.

B. M. Duggar, Professor of Physiological and Economic Botany, University of Wisconsin.

C. E. Allen, Professor of Botany, University of Wisconsin.

S. C. Brooks, Professor of Zoölogy, University of California.

Ivey F. Lewis, Professor of Biology, University of Virginia.

Wm. J. Robbins, Professor of Botany, University of Missouri.

II. INSTRUCTION.

William Randolph Taylor, Assistant Professor of Botany, University of Pennsylvania.

Hugh P. Bell, Associate Professor of Botany, Dalhousie University.

James P. Poole, Professor of Evolution, Dartmouth College.

LIBRARY.

Priscilla B. Montgomery (Mrs. Thomas H. Montgomery, Jr.) Librarian.

Gwendolen Hughes, Assistant Librarian.

Deborah Lawrence, Secretary.

Mary Rohan, Clerk.

CHEMICAL SUPPLIES.

Oliver S. Strong, Professor of Neurology, and Neuro-Histology, Columbia University, *Chemist.*

APPARATUS ROOM.

Samuel E. Pond, Assistant Professor of Physiology, Medical School, University of Pennsylvania, *Custodian of Apparatus.*

SUPPLY DEPARTMENT.

George M. Gray, Curator.
Assistant Curator: ——— ———.
John J. Veeder, Captain.
E. M. Lewis, Engineer.
A. W. Leathers, Head of Shipping Department.
A. M. Hilton, Collector.
J. McInnis, Collector.

F. M. MacNaught, Business Manager.
Herbert A. Hilton, Superintendent of Buildings and Grounds.
Thomas Larkin, Superintendent of Mechanical Department.
Lester F. Boss, Mechanician.
William Hemenway, Carpenter.
Arnold H. Bisco, Storekeeper and Head Janitor.

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2. INVESTIGATORS AND STUDENTS, 1928.

Independent Investigators

Abramson, H. A., Cornell University Medical College.
Addison, William H. F., Professor of Normal Histology and Embryology, University of Pennsylvania.
Allee, W. C., Associate Professor of Zoölogy, University of Chicago.
Armstrong, Philip B., Instructor in Anatomy, Cornell University Medical College.
Arndt, Charles H., Director, Coffee Experiment Station, Haiti.
Austin, Mary L., Lecturer, Barnard College.
Baitsell, George A., Associate Professor of Biology, Yale University.
Bard, Philip, Instructor in Physiology, Harvard University Medical School.
Barron, E. S. Guzman, Voluntary Assistant in Medicine, Johns Hopkins University.
Bazett, Henry C., Professor of Physiology, University of Pennsylvania.
Bell, Hugh P., Associate Professor of Botany, Dalhousie University.
Bennitt, Rudolf, Associate Professor of Zoölogy, University of Missouri.
Bernstein, Felix, Professor, University of Gottingen, Germany.
Bigelow, Robert P., Professor of Zoölogy and Parasitology, Massachusetts Institute of Technology.
Bishop, Mabel, Professor of Zoölogy, Hood College.
Bissonnette, T. Hume, Professor of Biology, Trinity College.

Blanchard, Kenneth C., Assistant Professor of Biochemistry, New York University.

Bliss, Sidney, Assistant Professor of Biochemistry, McGill University.

Blumenthal, Reuben, Graduate Student, University of Pennsylvania.

Boone, Ilsley, Brown University.

Bowen, Robert H., Associate Professor of Zoölogy, Columbia University.

Breitenbecher, Joseph K., Fellow in Zoölogy, McGill University.

Bridges, Calvin B., Columbia University.

Brien, Paul, Professor, University of Brussels.

Brinley, Floyd J., University of Pennsylvania.

Bronfenbrenner, J., Associate Member, Rockefeller Institute.

Brooks, Matilda M., Research Associate in Biology, University of California.

Brooks, S. C., Professor of Zoölogy, University of California.

Brown, Alice L., Assistant in Pathology, Cornell University Medical College.

Buchanan, J. William, Assistant Professor of Biology, Yale University.

Budington, R. A., Professor of Zoölogy, Oberlin College.

Burns, Robert K., Assistant Professor of Zoölogy, University of Cincinnati.

Butler, Elmer G., Instructor in Comparative Anatomy, Princeton University.

Calkins, Gary N., Professor of Protozoölogy, Columbia University.

Campbell, C. J., Associate Professor of Physiology, College of Medicine, Syracuse University.

Carey, Cornelia L., Instructor in Botany, Barnard College.

Carothers, Eleanor, Lecturer in Zoölogy, University of Pennsylvania.

Carpenter, Russell L., Assistant Instructor, Harvard University.

Cattell, Ware, Research Fellow in Biophysics, Memorial Hospital.

Chambers, Robert, Professor of Biology, New York University.

Chidester, F. E., Professor of Zoölogy, University of West Virginia.

Christie, Jesse R., Associate Nematologist, U. S. Department of Agriculture.

Clark, Eleanor L., Voluntary Investigator, University of Pennsylvania.

Clark, Eliot R., Professor of Anatomy, University of Pennsylvania Medical School.

Clarke, Miriam F., Instructor in Physiology, College for Women, Western Reserve University.

Clowes, G. H. A., Director, Lilly Research Laboratory, Eli Lilly & Co.

Cobb, N. A., Technologist and Nematologist, U. S. Department of Agriculture.

Coe, Wesley R., Professor of Biology, Yale University.

Cole, Elbert C., Assistant Professor, Williams College.

Cole, Kenneth, Research Fellow in General Physiology and Physics, Harvard University.

Conklin, Edwin G., Professor of Biology, Princeton University.

Copeland, Manton, Professor of Biology, Bowdoin College.

Cowdry, E. V., Associate Member, Rockefeller Institute.

Cowles, R. P., Associate Professor, Johns Hopkins University.

Crabb, Edward D., Instructor in Zoölogy, University of Pennsylvania.

Curtis, W. C., Professor of Zoölogy, University of Missouri.

Dawson, J. A., Instructor in Zoölogy, Harvard University.

Demerec, M., Carnegie Institution, Cold Spring Harbor.

Dobzhansky, Theodosius, University of Leningrad, Russia.

Dolley, William L., Professor of Biology, University of Buffalo.

Donaldson, Henry H., Member, The Wistar Institute of Anatomy and Biology.

Dreyer, Nicholas B., Lecturer in Pharmacology, McGill University.

Duggar, B. M., Professor of Physiological and Applied Botany, University of Wisconsin.

Edwards, D. J., Associate Professor of Physiology, Cornell University Medical College.

Emmart, Emily W., Graduate Student, The Johns Hopkins University.

Fish, Harold D., Research Associate in Laboratory of Mammal Genetics, University of Michigan.

Flexner, Louis B., Johns Hopkins Hospital.

Fogg, John M., Instructor in Botany, University of Pennsylvania.

Frank, Richard L., Graduate Student, Yale University.

Fry, Henry J., Assistant Professor of Biology, Washington Square College.

Gardiner, Mary S., Instructor, Bryn Mawr College.

Garrey, Walter E., Professor of Physiology, Vanderbilt University Medical School.

Gates, Frederick L., Associate Member, The Rockefeller Institute for Medical Research.

Glaser, Otto, Professor, Amherst College.

Goodrich, Hubert B., Professor of Biology, Wesleyan University.

Graham, John Y., Professor of Biology, University of Alabama.

Grant, Madeleine P., Assistant Professor, Mt. Holyoke College.

Grave, Benjamin H., Professor of Zoölogy, Wabash College.

Grave, Caswell, Professor of Zoölogy, Washington University.

Gray, James, Lecturer in Experimental Zoölogy, University of Cambridge.

Green, Arda A., Harvard University Medical School.

Grundfest, Harry, University Fellow, Columbia University.

Guthrie, Mary J., Associate Professor of Zoölogy, University of Missouri.

Hance, Robert T., Head of Department of Zoölogy, University of Pittsburgh.

Harnly, Morris H., Instructor, New York University.

Hartline, H. K., Department of Physics, Johns Hopkins University.

Harvey, E. Newton, Professor of Physiology, Princeton University.

Harvey, Ethel B., Princeton University.

Haywood, Charlotte, Assistant Professor of Physiology, Vassar College.

Hazen, Tracy E., Assistant Professor of Botany, Columbia University.

Hecht, Selig, Associate Professor of Biophysics, Columbia University.

Heilbrunn, L. V., Assistant Professor of Zoölogy, University of Michigan.

Hinrichs, Marie A., Research Associate, University of Chicago.

Hou, H. Chuan, Assistant in Physiology, Peking Union Medical College.

Howard, Harvey J., Professor of Ophthalmology and Director of the McMillan Eye Institute, Washington University, School of Medicine.

Howe, H. E., Editor, Industrial and Engineering Chemistry.

Howland, Ruth B., Assistant Professor of Biology, Washington Square College.

Huettner, Alfred F., Associate Professor, New York University.

Hughes, Thomas P., Associate in Pathology and Bacteriology, Rockefeller Institute.

Imai, Yoshitaka, Columbia University.

Izquierdo, J. Joaquin, Professor of Physiology, Escuelu Medico Militar.

Jacobs, M. H., Professor of General Physiology, University of Pennsylvania.

Jennings, H. S., Professor and Director of Zoölogical Laboratory, Johns Hopkins University.

Johlin, J. M., Associate Professor of Bio-Chemistry, Vanderbilt Medical School.

Jones, Edgar P., Graduate Assistant, University of Pittsburgh.

Just, E. E., Professor of Zoölogy, Howard University.

Kaan, Helen W., Associate Professor of Zoölogy, Wheaton College.

Keefe, Rev. Anselm M., Professor of Biology, St. Norbert College.

Knower, Henry McE., Professor of Anatomy, University of Alabama.

Knowlton, Frank P., Professor of Physiology, College of Medicine, Syracuse University.

Kozelka, A. W., Instructor, University of Pittsburgh.

Kropp, Benjamin, Instructor in Zoölogy, Harvard University.

Lackey, James B., Instructor, Washington Square College.

Lancefield, D. E., Assistant Professor, Columbia University.

Lancefield, Rebecca C., Assistant in Bacteriology, Rockefeller Institute.

Lewis, Ivey F., Professor of Biology, University of Virginia.

Lillie, Frank R., Chairman of the Department of Zoölogy, University of Chicago.

Lillie, Ralph S., Professor of General Physiology, University of Chicago.

Loeb, Leo, Professor of Pathology, Washington University Medical School.

Lucas, Catherine L. T., Research Fellow, Yale University.

Lucas, Miriam Scott, University of Pennsylvania.

Lucke, Baldwin, Associate Professor of Pathology, University of Pennsylvania.

Lynch, Ruth Stocking, Instructor in Zoölogy, The Johns Hopkins University.

MacCreight, Jean, Instructor, University of Pittsburgh.

McCutcheon, Morton, Assistant Professor of Pathology, University of Pennsylvania.

MacDougall, Mary Stuart, Head of Biology Department, Agnes Scott College.

McGlone, Bartgis, Instructor, University of Pennsylvania.

Manwell, Reginald D., Professor of Biology, West Virginia Wesleyan College.

Marshall, E. K., Jr., Professor of Physiology, Johns Hopkins Medical School.

Martin, Earl A., Assistant Professor of Biology, College of the City of New York.

Mast, S. O., Professor of Zoölogy, Johns Hopkins University.

Metcalf, Maynard M., Research Associate of Zoölogy, Johns Hopkins University.

Metz, Charles W., Member of Staff, Carnegie Institution of Washington.

Michaelis, Leonor, Resident Lecturer, Johns Hopkins University.

Mitchell, Philip H., Professor of Physiology, Brown University.

Mitchell, William H., Harvard University.

Morgan, Lilian V., Columbia University.

Morgan, T. H., Professor of Experimental Zoölogy, Columbia University.

Morgulis, Sergius, Professor of Biochemistry, University of Nebraska.

Morrison, Thomas F., Instructor, Princeton University.

Mudd, Stuart, Assistant Professor of Experimental Pathology, University of Pennsylvania.

Murray, Margaret R., University of Chicago.

Nakajima, Hisashi, Assistant in Bacteriology, Government Institute for Infectious Diseases, Tokyo, Japan.

Natarajan, C. V., Johns Hopkins School of Hygiene and Public Health.

Nishibe, Masujiro, Staff of the Government, Institute for Infectious Diseases, Tokio Imperial University.

Noble, G. Kingsley, Curator, American Museum of Natural History.

Nonidez, Jose F., Associate in Anatomy, Cornell University Medical College.

Osterhout, W. J. V., Member, Rockefeller Institute for Medical Research.

Packard, Charles, Assistant Professor of Zoölogy, Institute of Cancer Research, Columbia University.

Page, Irvine H., Eli Lilly & Co.

Pankratz, D. S., Instructor in Anatomy, University of Kansas.

Parker, G. H., Director, Harvard Zoölogical Laboratory, Harvard University.

Parmenter, Charles L., Assistant Professor, University of Pennsylvania.

Payne, Nellie M. C., University of Pennsylvania.

Pelluet, Dixie, Teachers' College, Murray, Kentucky.

Pinney, Mary E., Professor of Zoölogy, Milwaukee-Downer College.

Plough, Harold H., Professor of Biology, Amherst College.

Plunkett, Charles R., Assistant Professor of Biology, New York University.

Pollister, Arthur W., Graduate Student, Columbia University.

Pond, Samuel E., Assistant Professor of Physiology, University of Pennsylvania, School of Medicine.

Poole, James P., Professor of Evolution, Dartmouth College.

Rand, Herbert W., Associate Professor of Zoölogy, Harvard University.

Redfield, Alfred C., Assistant Professor of Physiology, Harvard University Medical School.

Redfield, Helen, Columbia University.

Reznikoff, Paul, Associate in Anatomy, Cornell University Medical College.

Richards, Alfred N., Professor of Pharmacology, University of Pennsylvania.

Richards, Oscar W., Assistant Professor of Biology, Clark University.

Richardson, Flavia L., Instructor in Zoölogy, University of Vermont.

Rogers, Charles G., Professor of Comparative Physiology, Oberlin College.

Sanders, Elizabeth P., Johns Hopkins University.

Sands, Jane, Professor of Physiology, Woman's Medical College of Pennsylvania.

Sayles, Leonard P., Instructor, Tufts College.

Schrader, Franz, Associate Professor, Bryn Mawr College.

Schrader, Sally-Hughes, Instructor in Biology, Bryn Mawr College.

Schultz, Jack, Columbia University.

Scott, Gordon H., Assistant, The Rockefeller Institute for Medical Research.

Severinghaus, Aura E., Instructor in Anatomy, Columbia University.

Shaftesbury, Archie D., Associate Professor of Zoölogy, North Carolina College for Women.

Shattuck, George E., Assistant, New York University.

Shlaer, Simon, Assistant, Columbia University.

Shull, A. Franklin, Professor of Zoölogy, University of Michigan.

Smith, George H., Biological Abstracts, University of Pennsylvania.

Sokoloff, Boris, Professor, University of Prague.

Speidel, Carl C., Associate Professor of Anatomy, University of Virginia.

Steele, Dewey G., Instructor in Biology, Yale University.

Stewart, Fred W., Pathologist, Rockefeller Institute for Medical Research.

Stockard, Charles R., Professor of Anatomy, Cornell University Medical College.

Stokey, Alma G., Professor of Botany, Mount Holyoke College.

Strong, Oliver S., Professor of Neurology and Neuro-Histology, Columbia University.

Stunkard, H. W., Professor of Biology, New York University.

Sturtevant, A. H., Columbia University.

Swett, F. H., Associate Professor of Anatomy, Vanderbilt Medical School.

Taft, Charles H., Jr., Graduate Student, College of Physicians and Surgeons.

Taylor, W. Randolph, Professor of Botany, University of Pennsylvania.

Tennent, David H., Professor of Biology, Bryn Mawr College.

Uhlenhuth, Eduard, Associate Professor of Anatomy, University of Maryland, Medical School.

Unger, W. Byers, Assistant Professor of Zoölogy, Dartmouth College.

Wang, Chi Chi, University of Pennsylvania.

Warren, Herbert S., Associate Professor of Zoölogy, University of Idaho.

Warren, Howard C., Stuart Professor of Psychology, Princeton University.

Weech, A. A., Instructor in Research Medicine, Johns Hopkins University.

Weill, Robert F., Assistant, University of Paris, Paris, France.

Weiss, Charles, Associate Professor of Experimental Bacteriology in Ophthalmology, Washington University.

Whitaker, Douglas M., Instructor in Physiology, Stanford University.

Whiting, Anna R., Professor of Biology, Pennsylvania College for Women.

Whiting, Phineas W., Associate Professor of Zoölogy, University of Pittsburgh.

Wieman, H. L., Professor of Zoölogy, University of Cincinnati.

Willey, Charles H., Instructor, New York University.

Williams, S. Culver, Instructor in Zoölogy, Brown University.

Williams, S. R., Professor of Physics, Amherst College.

Wilson, Edmund B., Professor of Zoölogy, Emeritus in Residence, Columbia University.

Wilson, J. W., Assistant Professor of Biology, Brown University.

Wolf, Ernst, University of Heidelberg, Heidelberg, Germany.

Wolf, E. Alfred, Instructor, Department of Zoölogy, University of Pittsburgh.

Woodruff, L. L., Professor of Protozoölogy, Yale University.

Young, Donnell B., Chairman, Department of Biology, University of Maine.

Young, William C., Research Assistant, University of Chicago.

Beginning Investigators.

Bailey, P. L., Jr., Graduate Student, Brown University.

Bangson, John S., Instructor in Biology, Berea College.

Barth, L. G., Graduate Assistant, University of Michigan.

Belkin, Morris, Harvard University.

Borquist, May, Research Fellow, Cornell University Medical College.

Bostian, Carey H., Graduate Assistant, University of Pittsburgh.

Bowling, Rachel, Assistant in Protozoölogy, Columbia University.

Bowman, Paul W., Instructor in Botany, George Washington University.

Carpenter, Esther, Fellow in Biology, Bryn Mawr College.

Carver, G. L., Professor of Biology, Mercer University.

Chase, Aurin M., Assistant, Amherst College.

Clark, L. B., Graduate Student, The Johns Hopkins University.

Coldwater, Kenneth, Graduate Assistant, University of Missouri.

Comegys, Margaret B., Columbia University.

Costello, Donald P., Assistant in Zoölogy, College of the City of Detroit.

Freeman, Leo B., University of Pennsylvania, Medical School.

Genther, Ida T., Assistant in Pathology, Washington University Medical School.

Gordon, Irving I., Research Assistant, Cornell University Medical School.

Goss, Charles M., Instructor in Anatomy, Yale University.

Graef, Irving, Volunteer Research Assistant, Cornell University Medical College.

Grafflin, Allan L., Johns Hopkins Medical School.

Hamilton, Lysbeth L., Graduate Assistant, University of Pittsburgh.

Hansen, Ira B., Graduate Assistant in Zoölogy, Wesleyan University.

Heath, Clark W., Massachusetts General Hospital.

Henderson, Jean T., Lecturer in Zoölogy, McGill University.

Hetherington, W. Alford, Assistant, Columbia University.

Hickman, Jane, Graduate Student, University of Missouri.

Holmes, Gladys E., Brown University.

Ignelzi, Marie A., Graduate Student, University of Pittsburgh.

Johnson, Percy L., Graduate Assistant, The Johns Hopkins University.
Keil, Elsa, Graduate Assistant in Biology, Brown University.
Keosian, John, Assistant in Biology, New York University.
Kinney, Elizabeth T., Graduate Student, Columbia University.
Lorberblatt, Isaac, Research Assistant, Harriman Research Laboratory, Brooklyn, New York.
Lucas, Alfred M., Instructor, Washington University.
Lu, Hwei-Ling, Research Worker, Memorial Hospital, New York City.
Lynch, Mary E., Teacher of Biology, Boston, Massachusetts.
McCardle, Ross C., Assistant, Brown University.
Marsland, Douglas A., Instructor in Biology, New York University.
Mather, Vera G., Cornell University Medical College.
Miller, Evelyn H., Graduate Student, University of Pennsylvania.
Miller, Ruth C., Graduate Student, Bryn Mawr College.
Montgomery, Hugh, Harvard University Medical School.
Morris, Helen S., Graduate Student, Columbia University.
Morrison, Mary E., University of Pennsylvania.
Mudd, Emily B. H., Henry Phipps Institute, University of Pennsylvania.
Mowery, May, Technical Assistant, Carleton College.
Nabrit, Samuel M., Brown University.
Nahm, Laura J., Instructor in Biology, Flat River Junior College.
Parpart, Arthur K., Instructor in Embryology, Amherst College.
Patterson, Ralph M., Graduate Student, University of Michigan.
Pollack, Herbert, Cornell University Medical College.
Preu, Paul W., Cornell University Medical College.
Raney, M. Hardie, Princeton University.
Ritter, Raymond, Graduate Assistant, University of Missouri.
Robertson, George M., Instructor in Evolution, Dartmouth College.
Root, Walter S., Assistant Instructor, University of Pennsylvania.
Rowe, Charlotte, New York University.
Rowell, Lyman S., Instructor, University of Vermont.
Schwartzbach, Saul, University of Maryland Medical School.
Schwarzkoff, Helen, Research Worker, Cornell University Medical College.
Sears, Mary, Radcliffe College.
Shoup, Charles S., Assistant in General Physiology, Princeton University.
Sichel, Ferdinand J. M., McGill University.
Smith, Helen B., Johns Hopkins University.
Snell, George D., Graduate Student, Harvard University.
Steinhardt, J., Assistant, Columbia University.
Stewart, Dorothy R., Graduate Student, University of Pennsylvania.
Struthers, Charlotte M., Instructor in Zoölogy, University of Pittsburgh.
Sumwalt, Margaret, Instructor in Physiology, Womens' Medical College of Pennsylvania.
Taylor, Jean Grant, Philadelphia, Pennsylvania.
Thompson, Helen, Graduate Assistant, University of Missouri.
Titlebaum, Albert, Instructor in Zoölogy, Columbia University.
Torvik, Magnhild M., Graduate Assistant, University of Pittsburgh.

Wilbar, Charles L., Jr., University of Pennsylvania.
Wilde, Frances M., Radcliffe College.
Williams, C. D., Buckhannon, West Virginia.
Winter, Charles A., Student Assistant, Johns Hopkins University.
Woods, Farris H., Instructor, University of Missouri.
Yamaguchi, Masamichi, Assistant Professor, Niigata Medical College, Japan.
Young, Roger A., Assistant Professor, Howard University.

RESEARCH ASSISTANTS 1928.

Apgar, Grace M., Research Assistant, University of Pennsylvania.
Armstrong, Eleanor F., Assistant, Cornell University Medical College.
Averell, Philip R., Research Assistant, Rockefeller Institute for Medical Research.
Buck, Louise H., Research Assistant, Columbia University.
Dalton, Albert J., Graduate Assistant, Wesleyan University.
Dobzhansky, Nathalie P., University of Leningrad.
Greene, Eunice C., Research Fellow in Anatomy, College of Medicine, Syracuse University.
Harnly, Marie L., Carnegie Institution, New York City.
Harrop, George A., Associate Professor of Medicine, Johns Hopkins Medical School.
Hoskins, Frances, Columbia University.
Ingalls, Elizabeth N., Research Assistant, Harvard University Medical School.
Keltch, Anna K., Lilly Research Laboratory, Eli Lilly Co.
Muckenfuss, Ralph S., Assistant, Department of Pathology and Bacteriology, Rockefeller Institute.
Oliphant, Joseph F., Wabash College.
Parpart, Ethel R., Amherst College.
Plunkett, Marie L., Research Assistant, New York University.
Reynolds, Sara Jane, Research Assistant, New York University.
Schauffler, William G., Princeton, New Jersey.
Scholl, Anna E., University of Pittsburgh.
Smith, Doreen, Research Assistant, Memorial Hospital.
Smith, Wilbur A., University of Pennsylvania, School of Medicine.
Tatnall, Charles R., Medical School, University of Pennsylvania.
TerLouw, Adrian L., Carnegie Institution of Washington, Cold Spring Harbor.
Wallace, Edith M., Carnegie Institution of Washington.
Weare, J. H., Research Assistant, Harvard University Medical School.

STUDENTS.

Botany.

Bulmer, Gladys, University of Pennsylvania.
Burr, Belle H., Wellesley College.
Butler, Margaret R., Assistant in Botany, Dalhousie University.

Button, Marion, Goucher College.
Faull, Anna F., Radcliffe College.
Francis, Dorothy S., Student, Radcliffe College.
Hartman, Grace L., High School Teacher, East Cleveland.
Harvey, Alice G., Dalhousie University.
Hindley, Sara R., Goucher College.
MacMurray, Mary T., Biology Teacher, Richmond Hill, New York.
Passmore, Sara F., Teacher of Botany, University of Pennsylvania.
Pope, Gladys A., Biology Instructor, Washington, D. C.
Rollins, Mary L., H. Sophie Newcomb Memorial College.
Roscoe, Muriel V., Professor of Biology, Acadia University.
Smith, Jane R., University of Pittsburgh.
Souder, Mary E., Wellesley College.

Embryology.

Abell, Richard G., Instructor in Biology, Hampton Institute.
Arnold, Norman K., Undergraduate Assistant, Wesleyan University.
Beck, Lyle V., Wabash College.
Butts, Helen E., Graduate Student, Brown University.
Cogan, David G., Student, Dartmouth College.
Coldwater, Kenneth B., Graduate Assistant, University of Missouri.
Driver, Ernest C., Research Assistant, University of Illinois.
Drumtra, Elizabeth, Assistant in Biology, Wilson College.
Duryee, William R., Yale University.
Fowler, Ona M., University of Michigan.
Furnas, Naomi D., Assistant in Biology, Rockford College.
Holman, Katherine K., Student, Dalhousie University.
Kirkwood, Elizabeth S., Student, Mount Holyoke College.
MacCalmont, Robert W., Jr., University of Pennsylvania.
MacLennan, Ronald F., Oberlin College.
Macmillan, Ruth E., Assistant in Histology and Embryology, Cornell University.
McMillion, Theodore M., Instructor in Biology, Geneva College.
McPherson, Maurita E., Elmira College.
Phillips, Paul L., Assistant in Anatomy, Cornell University Medical College.
Rifenburg, Sumner A., Instructor in Zoölogy, Purdue University.
Robinson, Janet, Assistant, Wellesley College.
Rugh, Roberts, Instructor, Lawrence College.
Sanders, James M., Assistant in Zoölogy, Illinois University.
Smelser, George K., Student, Earlham College.
Smith, Septima C., Assistant Professor of Biology, University of Alabama.
Smith, Suzanne G., Graduate Assistant, Oberlin College.
Sun, Tson Pen, Instructor in Comparative Anatomy of Vertebrates, Southwestern University, China.
Ufford, Elizabeth H., Bryn Mawr College.
Wright, Lennel I., Instructor in Histology and Embryology, Kansas University.

Physiology.

von Ammon, Winona, Swarthmore College.
Binns, Dorothy A., Western Reserve University.
Goddard, Verz R., Instructor in Physiology, Wellesley College.
Graubard, Marc A., Laboratory Assistant, Columbia University.
Harris, Albert H., Student, Harvard University Medical School.
Izquierdo, J. J., Professor of Physiology, Escuela Medico Militar, Mexico City.
McGoun, Ralph C., Jr., Assistant in Biology, Amherst College.
Panton, Jean, Instructor, University of Toronto.
Reynolds, S. R. M., Instructor in Physiology—Zoölogy, Swarthmore College.
Robb, Robert C., Research Scholar, Bussey Institution.
Schweizer, Malvina, Assistant Instructor, New York University.
Spivack, David, Student, Rutgers College.
Wald, George D., Assistant Instructor in Biology, New York University.
Warren, Charles O., Assistant in Biology, New York University.
Waterman, Allyn J., Oberlin, Ohio.

Protozoölogy.

Baker, Clinton L., Professor of Biology, Millsaps College.
Biddle, Russell L., Graduate Assistant, New York University.
Frew, Priscilla E., Instructor in Comparative Anatomy, New York University.
Geiman, Quentin M., Teacher, Lansdale, Pennsylvania.
Goldin, Oscar, 21 East 110 Street, New York City.
Harding, Pearl M., East Longmeadow, Massachusetts.
Hook, Sabra J., Assistant in Zoölogy, Barnard College.
Hutchinson, George A., Instructor, University of Rochester.
Lewis, Elsie M., Graduate Student, Columbia University.
Nishibe, Masujiro, Associate Pathologist, Tokyo Imperial University.
Patterson, Mabelle R., Columbia University.
Skinker, Mary S., Head of Department of Biology, Pennsylvania College. for Women.
Smith, Willie W., Assistant in Biology, New York University.
Stabler, Robert M., Student, University of Pennsylvania.
Sturdivant, Harwell P., Columbia University.
Wolff, Walter H., Columbia University.

Invertebrate Zoölogy.

Apgar, Virginia, Student, Mt. Holyoke College.
Baier, Joseph G., Jr., Assistant in Zoölogy, Rutgers University.
Barsony, Mrs. L. M., New York University.
Brown, Margaret E., Teacher of Biology, Flora Macdonald College.
Caldwell, Lucile J., Assistant Instructor, Agnes Scott College.
Carney, Beatrice M., University of Buffalo.
Chapman, Alfred N., Williams College.
Church, Frances, Instructor in Zoölogy, Carleton College.
Clark, Elsie S., Student, Radcliffe College.

Cordes, Warren P., Student, Colgate University.
Coulter, Mrs. John C., University of South Carolina.
Cranston, William J., Jr., Hamilton College.
DuShane, Graham P., Wabash College.
Findlay, Martha S., Graduate Student, Radcliffe College.
Garrison, Walter E., Student, DePauw University.
Gaylor, Earl L., Jr., Wesleyan University.
Gooch, Marjorie, Technician, Rockefeller Institute.
Gray, Nelson Milton, Student, McGill University.
Haight, Catherine L., Teacher of Biology, Boston Public Schools.
Herber, Elmer C., Assistant Instructor, University of Pennsylvania.
Herrick, Earl H., Austin Teaching Fellow, Harvard University.
Hughes, Gwladys F., North Forest Park, Baltimore, Maryland.
Johnson, Alfhild J., Assistant, Wellesley College.
Johnstone, Emma J., Johns Hopkins University.
Keyser, Walter LeRoy, University of Pennsylvania.
Lewis, Katharine, Mount Holyoke College.
MacFarlane, Constance I., Student, Dalhousie University.
Macauley, Aileen J., Student, Dalhousie University.
Magoun, Horace W., Rhode Island State College.
Miller, Eileen M., Instructor, New Jersey College for Women.
O'Neil, Helen D., University of Minnesota.
Parks, Elizabeth K., Mount Holyoke College.
Pappenheimer, A. M., Jr., Harvard University.
Pearl, Ruth D., Wellesley College.
Pierson, Bernice F., Western Reserve University.
Pitts, Robert F., Butler College.
Redmond, Willie B., Laboratory Instructor, Emory University.
Reid, Marion A., Boston University, Medical School.
Rheinberger, Margaret B., Smith College.
Robertson, C. W., Assistant, Southwestern.
Schneider, Elizabeth M., Johns Hopkins University.
Scribner, Edith I., High School Teacher, East Cleveland, Ohio.
Shaw, C. Ruth, Instructor, University of Kansas.
Slifer, Eleanor H., Graduate Student, University of Pennsylvania.
Smith, Ernest V., Jr., Wabash College.
Stanton, Elizabeth, Goucher College.
Stone, Raymond G., Instructor, University of Missouri.
Street, Sibyl F., Student, Vassar College.
Taylor, Grace H., Teacher of Biology, Randolph-Macon.
Todd, Virginia Lee, Laboratory Assistant, Washington University.
Vanijvadhana, Subhajaya, Student, Princeton University.
Violette, Homer N., Student, Yale University.
Walker, Roland, Oberlin College.
Wallace, Marjorie A., Goucher College.
White, William E., Teaching Fellow in Biology, University of Alabama.
Winkleman, Elvene A., Graduate Assistant, Washington University.
Yntema, Chester L., Graduate Student, Yale University.

3. TABULAR VIEW OF ATTENDANCE.

	1924	1925	1926	1927	1928
INVESTIGATORS—Total	194	207	252	294	323
Independent	124	135	156	209	217
Under Instruction	70	72	84	57	81
Research Assistants			12	28	25
STUDENTS—Total	134	132	141	141	133
Zoölogy	50	54	56	57	57
Protozoölogy	17	17	19	17	16
Embryology	29	29	28	32	29
Physiology	18	19	18	19	15
Botany	20	13	20	16	16
TOTAL ATTENDANCE	328	339	393	435	456
Less persons registered as both students and investigators			8	1	2
			385	434	454
INSTITUTIONS REPRESENTED—Total	110	112	119	111	111
By investigators	69	74	84	89	80
By students	68	65	60	63	66
SCHOOLS AND ACADEMIES REPRESENTED					
By investigators	–	1	–	1	1
By students	–	4	4	4	1
FOREIGN INSTITUTIONS REPRESENTED					
By investigators	–	–	17	15	13
By students	–	–	3	8	8

4. SUBSCRIBING AND COOPERATING INSTITUTIONS 1928.

Amherst College
American Museum of Natural History
Antioch College
Barnard College
Berea College
Bowdoin College
Brown University
Bryn Mawr College
Butler College
Carleton College
Carnegie Institution, Cold Spring Harbor
Carnegie Institution of Washington
Columbia University
Cornell University Medical College
Dalhousie University
Dartmouth College
DePauw University
Duke University
Elmira College
T. W. Evans Dental Museum and School of Dentistry
General Education Board

Goucher College
Hamilton College
Harvard University
Harvard University Medical School
Howard University
Industrial & Engineering Chemistry, of the American Chemical Society
International Education Board
Johns Hopkins University
Johns Hopkins University Medical School
Eli Lilly & Co.
McGill University
Massachusetts Institute of Technology
Memorial Hospital of N. Y. City
Mount Holyoke College
National Research Council
New York University
North Carolina College for Women
Oberlin College
Princeton University
Radcliffe College
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University
Smith College
Sophie Newcomb College
Southwestern
Swarthmore College
Tufts College
United States Department of Agriculture
University of Alabama
University of Buffalo
University of Chicago
University of Cincinnati
University of Illinois
University of Maryland Medical School
University of Michigan
University of Minnesota
University of Missouri
University of Pennsylvania
University of Pennsylvania Medical School
University of Pittsburgh
University of Rochester
University of Rochester Medical School
University of Vermont
University of Virginia
University of Wisconsin
Vanderbilt University Medical School
Vassar College
Wabash College
Washington University
Washington University Medical School
Wellesley College
Wesleyan University
Western Reserve University
Wistar Institute of Anatomy and Biology
Womens College of Pennsylvania
Yale University

SCHOLARSHIP TABLES.

Ida H. Hyde Scholarship of the University of Kansas.

Lucretia Crocker Scholarships for Teachers in Boston.

Scholarship of $100.00 supported by a friend of the Laboratory since 1898.

The Edwin S. Linton Memorial Scholarship of Washington and Jefferson College.

The Bio Club Scholarship of the College of the City of New York.

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5. EVENING LECTURES, 1928.

Friday, June 29,
Dr. H. C. Bazett .... "Some Effects of Temperature Changes on Living Animals, Particularly on Man."

Friday, July 6,
Dr. G. Kingsley Noble .... "On Santo Domingan Trails." Illustrated.

Friday, July 13,
Dr. M. Demerec .... "Behavior of Mutable Genes."

Friday, July 20,
Dr. E. V. Cowdry .... "Diseases Caused by Invisible and Filterable Viruses."

Wednesday, July 25 (Special Lecture)
Dr. Harvey J. Howard .... "Social, Political, and Medical Experiences of an American Physician with Manchurian Bandits." Illustrated.

Friday, July 27,
The William Thompson Sedgwick Memorial Lecture, delivered by Dr. E. G. Conklin . "Problems of Development."

Friday, August 3,
Dr. A. G. Huntsman .... "The Oceanic Problem."

Friday, August 10,
Mr. J. Gray .... "The Mechanism of Ciliary Action."

Friday, August 17,
Dr. A. N. Richards .... "Experiments on the Elimination of Certain Dyes by the Kidney."

Tuesday, August 21,	
Dr. H. U. Sverdrup	"Experiences among Siberian Natives and from the Drift Ice." Illustrated.
Friday, August 24,	
Dr. Felix Bernstein	"Heredity and Human Races."

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6. SHORTER SCIENTIFIC PAPERS, 1928.

Tuesday, July 3,	
Dr. Charles Packard	"Biological Measurement of X-Rays."
Dr. E. Newton Harvey	"High Frequency Sound Waves and Their Biological Effects."
Dr. Kenneth Cole	"Electrical Impedance of Suspensions of Arbacia Eggs."
Tuesday, July 10,	
Dr. D. E. Lancefield	"Crosses of two Races of Drosophila obscura of Nearly the Rank of Physiological Species."
Dr. Calvin B. Bridges	"The Chromosomal Complex of Drosophila melanogaster."
Dr. P. W. Whiting	"Production of Mutations by X-Rays in Habrobrachon."
Tuesday, July 17,	
Dr. Sergius Morgulis	"Destruction of Catalase by Hydrogen Peroxide."
Dr. A. P. Mathews	"The use of Crotalus Venom in Analyzing Blood Clotting."
Drs. L. Michaelis and L. Flexner	"Reduction Potential of Cystein."
Tuesday, July 24,	
Dr. W. R. Taylor	"Algal Floras of the Western Atlantic."
Dr. I. F. Lewis	"Floristic Succession in the Dismal Swamp of Virginia."
Dr. B. M. Duggar	"Further Studies on the Properties of the Virus of Tobacco Mosaic."
Tuesday, July 31,	
Dr. Henry J. Fry	"The So-called Central Bodies in Fertilized Echinoderm Eggs."

Dr. Harold H. Plough .... "Differentiation in the Eggs of Echinus and Strongylocentrotus at the Time of the First Cleavage."

Dr. E. Eleanor Carothers .... "The Maturation Divisions and Segregation."

Tuesday, August 7,

Dr. Margaret Murray .... "Changes in Planarian Cells Cultivated in Vitro."

Dr. S. O. Mast .... "Changes in the Water Content of Ameba."

Dr. G. A. Baitsell .... "Coagulation in Relation to Tissue Formation."

Tuesday, August 14,

Dr. A. C. Redfield .... "Rôle of the Blood in Asphyxiation of the Squid."

Dr. W. E. Garrey .... "The Basal Leucocyte Count and Its Physiological Variations."

Dr. E. K. Marshall and Mr. Allan R. Grafflin .... "Structure and Function of the Kidney of the Goose-Fish."

Monday, August 20,

Dr. A. Franklin Shull ...... "Developmental Response to Light and Temperature in Aphids."

Dr. Caswell Grave .... "Light as a Factor in the Metamorphosis of the Larva of Ascidians."

Dr. Marie A. Hinrichs .... "Ultraviolet Radiation: Stimula- and Inhibition."

Dr. E. A. Wolf .... "An Ultraviolet Microradiator, Home-made and Inexpensive."

Tuesday, August 28,

Mr. J. R. Christie .... "The Influence of Environment on the Determination of Sex in the Mermithidae."

Dr. T. H. Bissonnette .... "A Case of Potential Freemartins in Cats."

Emily B. H. Mudd, Dr. Stuart Mudd, and Miss Anna K. Kelch .... "The Probable Relation of Echinid Spermagglutinins to Penetration of the Sperm into the Egg."
Dr. E. D. Crabb .... "Twinning in Fresh Water Snails."

Wednesday, August 29,
Dr. Philip M. Mitchell ...... "Factors Controlling the Potassium Content of Cells."
Mr. K. Blanchard .... "The Carbohydrate and Nucleoprotein Fractions of the Arbacia Egg."
Dr. J. M. Johlin .... "The Interfacial Adsorption as a Factor in the Clotting of Blood."
Miss Mary Morrison .... "Differences in the Physiological Properties of Several Commercial Preparations of Sodium Chloride."

Friday, August 31,
Dr. Robert Chambers .... "The Constancy of the Intraprotoplasmic pH."
Mr. Herbert Pollack .... "Calcium Ions in Protoplasm."
Doctors Baldwin Lucke and Morton McCutcheon .... "The Effect of Valence on Cellular Permeability to Water."

Monday, September 3,
Dr. Manton Copeland .... "Conditioned Reflexes in Nereis."
Dr. J. W. Wilson .... "Dominance in Planarian Regeneration."
Dr. Eduard Uhlenhuth and Mr. S. Schwartzbach ...... "Rôle of the Hypophysis in the Function of the Thyroid Gland."

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BIOLOGICAL BULLETIN

UROLEPTUS HALSEYI, N. SP. I. THE EFFECT OF ULTRA-VIOLET RAYS.

GARY N. CALKINS,
Columbia University, New York City.

Twelve years ago I found an organism which I described as a variety of *Uroleptus mobilis* Engelmann.[1] The origin of the culture which had been standing in the laboratory for four months was unknown. The organism was cultivated and studied for a period of ten years by the isolation culture method. It is evidently much more rare than *Uroleptus rattulus* or *U. pisces* for I have been unable to find any reference to it since its discovery by Engelmann in 1862.

Late in the fall of 1928 Mr. H. R. Halsey called my attention to a ciliate which he believed to be *Uroleptus mobilis* which he had found in one of the general class cultures. He had collected the material from a pond in Westchester County where sphagnum, *Myriophyllum*, etc., were abundant. The organism was present in great abundance and study soon showed that it was not *U. mobilis* but a closely related new species which I am very glad to name after my friend and assistant—*Uroleptus Halseyi*.

The two species are quite similar in general character and appearance and may be mistaken easily one for the other (Figs. 1, 2).

Engelmann's original description of *Uroleptus mobilis* runs as follows:

Body form constant; plastic; circular in section; about twelve times longer than broad; tapering gradually to a broadly pointed posterior end. Lateral cilia equally long throughout. With six elongate nuclei. This species which came from the Boticzbach near Prag, is distinguished from the other species of *Uroleptus* recently described by Stein, by the constant presence of six nuclei arranged one behind the other. It stands close to Stein's *Uroleptus rattulus* but, unlike this species, it possesses no sharply pointed posterior end, and the lateral cilia are equally long on the tail and body. The adoral row of cilia (adoral zone) occupies about one ninth of the total body length, with an undulating membrane fastened on its inner side; a peristome field appears to be entirely absent, or at least extremely narrow. Whether, as in other *Uroleptus* species, two longitudinal rows of fine ventral cilia are present, I could not make out since the animal is very lively and inclined to creep about, snake-like, between plant remains. Our species moreover, which appeared in great numbers, measured on the average 0.30 mm. All specimens were about of the same size (*loc. cit.*, p. 386).

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[1] *Uroleptus mobilis* Engelmann. I. History of the Nuclei during Division and Conjugation. Jour. Expt. Zoöl., Vol. 27, 1919.

The New York type of this species does not agree in all details with this original description as shown by the following account:

"Body form constant, plastic, circular in section; about 10.5 times longer than broad and tapering gradually to a broadly pointed posterior end. The posterior end is permanently curved towards the ventral side. (Engelmann does not mention this curvature but represents it in his figure.) The lateral cilia are long and distinct and sparsely distributed in straight rows running from end to end and around the posterior end of the body. Owing to density of the protoplasm the ventral cilia cannot be made out on the living organism but in cross sections it is evident that three rows of ventral cilia are present, and that they are finer and shorter than the lateral cilia. The frontal cirri, three in number, are placed in an oblique row at the extreme anterior end of the ventral surface. The terminal cilia of the ventral rows are conspicuous at the anterior end and give the impression of five or six frontal cirri. The peristome occupies about one sixth of the entire length of the body and this region is slightly flattened. The peristome is very narrow with an obliquely curved row of powerful pyramidal membranelles on the left side, but almost filling the peristomial area. The right margin is sharply cut with a narrow undulating membrane inserted between it and the adoral zone. The macronuclei are eight in number, and, in the vegetative stages, each possesses a typical nuclear cleft. The micronuclei vary in number from two to six; they are minute and homogeneous. The contractile vacuole is single, spherical, and lies in the center of the body near the dorsal surface. Length of the vegetative forms varies from 140 to 165 μ and the diameter at the center of the body from 11 to 17 μ. The cysts are spherical and the cyst wall smooth with an average diameter of 32.7 μ. (Calkins, 1919, p. 294.)

There are three points in which these descriptions of the supposedly same species do not agree. These are: (1) the length of the organism (Engelmann's 300 μ, the New York variety 158 μ; (2) the number of macronuclei (Engelmann 6, the New York variety 8); and (3) the position of the contractile vacuole (Engelmann, anterior third of body, the New York variety center of the body). Despite these differences however, it was decided to regard the New York form as an American variety of the European species. While this decision was probably an error in judgment it is too late now to propose a change and I shall refer to it here simply as the New York variety.

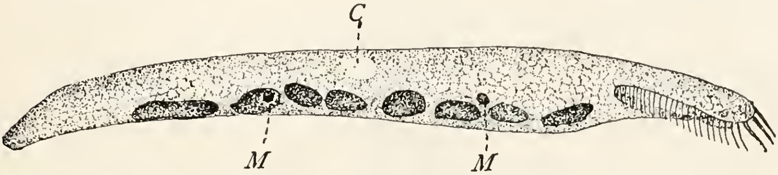


Fig. 1. *Uroleptus mobilis* Engel. New York variety. Cilia not shown. c = contractile vacuole; m = micronucleus. × 650.

Uroleptus Halseyi differs from both *Uroleptus mobilis* and the New York variety although superficially it resembles both (Fig. 2). The body form is constant, plastic, and circular in cross section and about nine times longer than it is broad. The posterior end tapers gradually to form a long sharp tail which is distinctly curved towards the ventral side. The body is much more wormlike than the New York variety and twists and doubles on itself in all directions as it forces its way into zoögloea masses or detritus of different kinds. The average length in fixed preparations is 163.2 μ and the average diameter is 18.7 μ but the organisms are capable of stretching out until the length is sixteen times

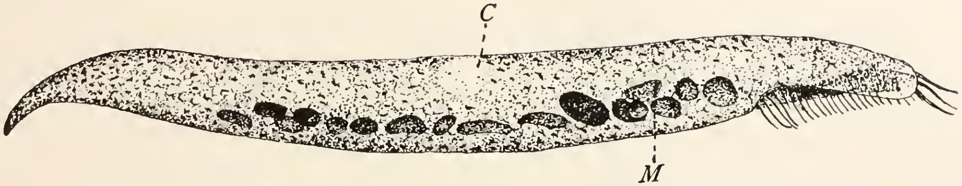


FIG. 2. *Uroleptus Halseyi*, n. sp. Cilia not shown. *c* = contractile vacuole; *m* = micronucleus. × 675

the diameter. The size therefore is highly variable and appears to be dependent upon the age of the culture and nature of the medium. The curvature of the tail is much more pronounced than in either of the other forms. The peristome is well marked and more conspicuous than in the New York variety and occupies from one sixth to one seventh of the total length of the body.

The descriptions given above of the three types of *Uroleptus* show very few structural differences of importance so far as the externals are concerned. Internally however, the differences are quite characteristic and well-marked. The cytoplasm of *Uroleptus Halseyi* is filled with iron hæmatoxylin staining granules especially after conjugation. These are interpreted as mitochondria of the protozoan type although in an unbroken cell they do not stain with Janus green. Nor do they stain with neutral red or any other vital dyes. They disappear after long exposure to xylol, turpentine or similar reagents and after a short treatment with acetic acid or other acids. They appear to be therefore, distinctly of a lipoid nature hence if not mitochondria they belong to the same group of cytoplasmic granules.

Other cytoplasmic granules stain vividly with neutral red; still others with methylene blue. The neutral red granules are more or less distributed throughout the endoplasm but are also present in lines running the length of the body on the surface. These are undoubtedly the excretory granules so-called which are characteristic of ciliates generally. The methylene blue granules are also arranged in longitudinal lines but are larger and more numerous than the neutral red granules.

As with the New York variety the number of macronuclei of *Uroleptus Halseyi* is occasionally eight, distributed in a linear series along the ventral half of the cell. This is the usual number in the New York variety, a larger number (nine to twelve) being exceptional. In mass cultures of the new species individuals are rarely found with eight macronuclei the great majority having from ten to sixteen while some individuals have as many as twenty-six. These nuclei take the usual chromatin dyes and give a positive reaction with the Feulgen method.

The micronuclei are very large and quite different from the

	Size.		Macronuclei.		Micronuclei.		Cont. vacuole.	Tail.
	Length.	Diam.	Number.	Size.	Number.	Size.		
U. mobilis	300μ	25μ	6	?	?	?	Anterior third	Inconspicuous
N. Y. Variety	158μ	15.6	8–12	3.8×8.3μ	2–6	2 ×2.5μ	Center—dorsal	Blunt, curved
U. Halseyi	163μ	18.7	8–26	3.2×8.4μ	1–2	2.8×9.3μ	“ “	Pointed “

micronuclei of the New York variety. After conjugation the organisms reorganize with two or occasionally three but in many adult individuals only one micronucleus can be found.

Uroleptus Halseyi is not as satisfactory for isolation cultures as the New York variety, but it grows well in mass cultures having a pH of 7.4 to 7.2. Its division and conjugation processes offer some interesting variations from those of the New York variety and these will be taken up in a later paper.

The essential differences in the three types of *Uroleptus* are given in the table on page 64.

Effect of Ultra-Violet Rays on *Uroleptus*.

A Cooper-Hewitt mercury lamp known as the Uviarc was used as the source of ultra-violet rays. The organisms to be exposed were contained in a flat Syracuse dish with a uniform depth of medium of three eighths of an inch. The surface of the medium was fixed at 23 centimeters from the source of light and the exposure was limited to exactly thirty seconds. Permanent preparations of the exposed *Uroleptus* were made ten minutes after exposure, twenty-four hours and forty-eight hours after. Sixty hours after exposure all *Uroleptus* individuals were dead although other species of Protozoa with the same exposure to the ultra-violet rays in the same container, continued to live. The *Uroleptus* material therefore had received a lethal dose but a dose that killed only after two days subsequent to exposure. Material was thus furnished which was suitable for a study of the effects of radiation on the cell constituents.

A culture of *Uroleptus Halseyi* with few dividing forms was used. This culture, four days before was actively dividing. Such actively dividing forms have an average length of 124.3 μ and an average diameter of 22.5 μ. The extremes were 180 x 20 and 120 x 25 μ thus showing a wide variation which is characteristic of actively dividing forms. In ordinary mass cultures the averages of length and breadth are 163.2 x 18.7. These measurements together with similar measurements of individuals killed ten minutes twenty-four hours and forty-eight hours after radiation are shown in the following table:

MEASUREMENTS OF 10 INDIVIDUALS EACH, IN MICRONS.

	Normal.				Exposure to ultra-violet rays.					
	Actively Dividing.		Vegetative.		10 Min. After.		24 Hours After.		48 Hours After.	
	Leng.	Diam.	Leng.	Diam.	Leng.	Diam.	Leng.	Diam.	Leng.	Diam.
	180	20	143	15	132	13	110	16	100	12
	112	26	150	16	150	12	111	16	90	15
	110	25	170	17	145	10	90	14	105	13
	123	24	192	19	145	14	82	13	112	12
	120	19	195	20	111	10	93	14	100	14
	116	22	160	20	142	12	69	20	110	10
	117	23	142	20	148	10	75	13	112	16
	129	27	160	20	110	11	85	14	103	14
	120	13	150	22	130	15	98	12	96	18
	116	26	170	18	122	9	78	13	100	17
Average	124.3	22.5	163.2	18.7	133.5	11.6	89.1	14.5	102.8	14.1

It is evident that one well-marked effect of the ultra-violet rays is a great reduction in size of the organism. This is manifested immediately after radiation as shown by the difference in average size of ten individuals taken at random, from 163.2 x 18.7 to 133.5 x 11.6. The decrease in size continues until at the end of twenty-four hours the average size is only 89.1 x 14.5 μ. Apparently a first effect, therefore is the loss of cell fluids, but there is a partial recovery during the second twenty-four hours, for the average size goes up to 102.8 x 14.1 μ. The organisms however are doomed for by the end of sixty hours after exposure they are all dead. Immediately after exposure they are active and apparently normal.

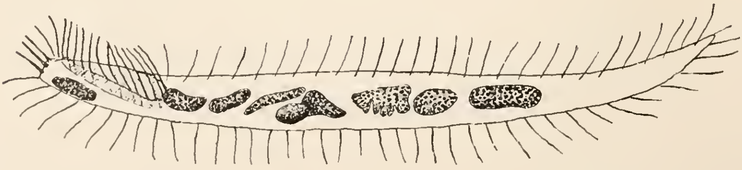
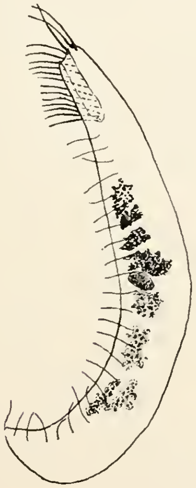


FIG. 3. *Uroleptus Halseyi.* Killed ten minutes after treatment with ultra-violet rays for 30 seconds. × 775.

Other effects of radiation are shown by the intra-cellular structures. The structures which we interpret as mitochondria are destroyed. They segregate in small granular masses which ultimately turn yellow. This leaves the cytoplasm strikingly clear and the nuclei in consequence are much easier to study.

In material fixed 10 minutes after exposure the nuclei have the normal linear arrangement. Chromatin and X granules are present and a normal picture is given both by the Borrel stain and the Feulgen reaction (Fig. 3).

Twenty-four hours after exposure there is a very different picture. Not only are the cells smaller but they are now uniformly sickle-shape, almost motionless, and have lost their characteristic caudal hooks (Fig. 5). The macronuclei are no longer in linear arrangement but are massed near the middle of the body as they are during the later phases of conjugation. The chromatin granules run together and the nuclei may be broken up into fragments which may remain together in a semblance of the former nucleus, or they may be dispersed in the cell. In either case there is an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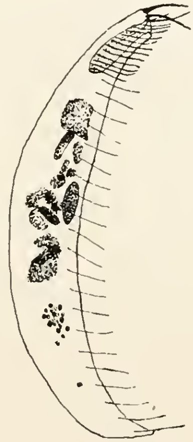
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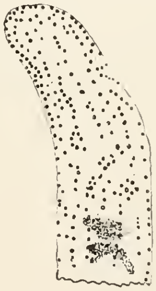
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Fig. 4. *Uroleptus Halseyi.* Killed 48 hours after treatment with ultra-violet rays for 30 seconds. × 775.

Fig. 5. *Uroleptus Halseyi.* Killed 24 hours after treatment with ultra-violet rays for 30 seconds. × 775.

Fig. 6. *Uroleptus Halseyi.* Posterior end of specimen killed 24 hours after treatment with ultra-violet rays for 30 seconds. × 1000.

entire disappearance of the nuclear membranes, the nuclei thus becoming emarginate and amorphous. The micronuclei are rarely intact and large portions are usually lacking as though bitten off.

Forty-eight hours after exposure there is no essential change in the picture. Spherical masses of yellow mitochondria (?) are present and are now found mainly in the posterior end of the cell (Fig. 4). Iron hæmatoxylin shows relatively enormous peripheral excretory granules in rows which run the entire length of the cell (Fig. 6). The micronuclei are usually much elongated and in consequence, appear like spindles but they are invariably homogeneous and with no trace of chromosomes or fibers.

The effects of ultra-violet rays on *Uroleptus Halseyi* may be briefly summarized as follows:

1. A reduction in size and a change in shape of the organism.

2. Movement is at first normal but slows down after six to eight hours. At 48 hours the organisms are quiet save for movement of the membranelles; at 60 hours they are dead.

3. Structures usually described as mitochondria disappear immediately after radiation. They collect in granular masses which ultimately turn yellow.

4. The macronuclei undergo granular degeneration and disintegrate.

5. The micronuclei become elongate and disfigured.

6. The characteristic Feulgen reaction is given at all stages indicating that the nucleic acid persists throughout.

7. The peripheral excretory granules are enlarged and apparently are more numerous.

8. An exposure of 30 seconds to ultra-violet rays produces no monsters; this dosage is lethal to *Uroleptus Halseyi,* but not lethal to many other types of Protozoa exposed at the same time.

FURTHER STUDIES UPON THE NEMATOCYSTS OF *MICROSTOMUM CAUDATUM.*

WM. A. KEPNER AND J. W. NUTTYCOMBE,
University of Virginia.

The nematocysts of *Microstomum caudatum* are those of hydra which the rhabdocœle has appropriated, after having eaten and digested the polyp. This was first shown by Martin (08), (14). He failed, however, to demonstrate how the *Microstomum* manipulated the nematocysts. Kepner (11) showed that the nematocysts were left naked, by the action of the gastric enzymes, within the enteric lumen (Fig. 1, *a*). The naked nematocysts were then taken up by the epithelial cells of the endoderm as particles of food are taken into food vacuoles by these cells (Fig. 1, *b*); but they are not ejected into the lumen of the enteron again as indigestible portions of the food are from food vacuoles. The nematocysts are then handed over to the mesenchyme by the endodermal cells. Here, for a time, they lie indifferently oriented within lacunæ (Fig. 1, *c*). Eventually a wandering mesenchymal cell appropriates each nematocyst. The mesenchymal cell becomes a cnidophage (Fig. 1, *d*). Meixner (23) published a paper in which he contends that the attending cells of the appropriated nematocysts are not cnidophages but the original cnidoblasts with nematocysts which have been carried all the way through the walls of the enteron and the mesenchyme of *Microstomum lineare* (Mull) to the surface of the rhabdocœle where they retain and discharge the nematocysts. Meixner's observations run quite counter to what we and the other students in this laboratory have repeatedly and without exception observed in *Microstomum caudatum*. We have seen the naked nematocysts lying within the enteron's lumen and also being carried through the endoderm into the mesenchyme in the living specimen as it was held immovable under a coverglass.

If Meixner should be correct in contending that the attending cells, which he and we see in *Microstomum,* be the original cnidoblasts, the situation is a very remarkable one; for we should then have isolated cells of hydra functioning in coöperation with the tissues of *Microstomum* in the discharge of nematocysts.

The manipulation of the nematocysts on the part of *Microstomum* is not an incidental phenomenon. In the first place the rhabdocœle does not accept hydras primarily for food. *Microstomum* is not like the Æolididæ, in this respect. Evans (22) says, "The Æolididæ all eat cœlenterata" (p. 453). In other words, cœlenterata constitute one of the regular types of food of these molluscs. This fact stands in sharp contrast with *Microstomum's* feeding upon hydra. When a "loaded" *Microstomum* is forced, through inanition, to feed upon hydra, it digests the tissues of the polyp and regurgitates the naked nematocysts after all the protoplasmic parts of the hydra have been digested. On the other hand, a well-fed *Microstomum*—one gorged with food, but lacking nematocysts—will make room for a hydra by ejecting some of its food and at once taking up the polyp. Thus it appears that *Microstomum,* unlike the Æolididæ, does not prey upon a cœlenterate primarily for food but for its nematocysts.

Since *Microstomum* thus seeks primarily the nematocysts of hydra and not its tissues, it must have some definite demand for these defensive and offensive structures of hydra. Kepner and Barker (24) have recorded much evidence of *Microstomum* using the nematocysts of hydra which it has appropriated.

The appropriation and use of hydra's nematocysts is, therefore, quite as definitely an instinct as is the appropriation of materials for and the building therewith of a nest on the part of a bird. Moreover, this is a complex instinct in that it involves the selective faculties of the *Microstomum* as a whole when it takes hydra, and of the endodermal cells when they take up and hand over to the mesenchyme only nematocysts and do not lay hold of other solid bodies and deliver them to the mesenchyme. The complexity of this instinct is further shown in that the cnidophages (or, if Meixner be correct, the cnidoblasts) carry the nematocysts to the surface in such a manner that the latter are uniformly distributed throughout the surface of the flatworm. Here the cnidophages act coöperatively. When once the nematocysts have been carried to the surface, the cnidophages function independently. When a "loaded" *Microstomum* is locally disturbed, only the nematocyst or nematocysts in the immediate vicinity of the disturbing factor will be shuttled to and fro in a threatening manner. If the disturbance be maintained, only the local nematocysts will be discharged.

Thus it is seen that in *Microstomum's* handling of the nematocysts of hydra we have a complex instinct. The factors for instincts are usually transmitted from generation to generation by the gametes. *Microstomum,* under laboratory conditions, seldom elaborates gametes and zygotes. In its natural habitat, one usually encounters them propagating asexually, but in the autumn zygotes appear.

In *Microstomum,* therefore, we recognized an opportunity to test the possibility of transmitting factors for an instinct, not through gametes, but, through somatic cells as the animal propagates asexually through fission, if a method for rearing *Microstomum* could be discovered.

Three years of casual effort netted only failure to rear *Microstomum* in the laboratory. Light and temperature conditions were variously controlled. The water in the culture dish was exchanged each day for water that had just been taken from the pond in which *Microstoma* lived. But with all no positive results were secured. Eventually it dawned upon us that perhaps the specimens needed sediment within which to be anchored, as it were, at rest. When we added a little dried sediment collected from the pond, we found that the animals no longer kept on the move, but spent much of the time lying quietly within the sediment and that thereafter we had little difficulty in carrying them as far as into the twenty-sixth asexual generation.

Having succeeded in finding a method of culturing them, we next attempted to determine whether the persistence of the complex instinct, involved in the handling of nematocysts by *Microstomum,* would be carried from asexual generation to asexual generation without experience with hydras.

This attempt was made by selecting wild individuals, that lacked nematocysts, from their natural habitat and establishing clones with them.

Clone "A′" was established April 30, 1926, from a wild individual that lacked nematocysts. By May 19, the seventh asexual generation had made its appearance. One of the two individuals of this generation was placed with a brown hydra. This hydra was eaten and its nematocysts appropriated. Similar results were obtained for an individual of the tenth asexual generation of this clone. A second clone "A" we succeeded in carrying into the twenty-sixth asexual generation before it died. One individual of the twenty-third asexual generation was placed with an hydra, which was later, in part, eaten and its nematocysts appropriated. Thus we found that *Microstoma* removed from experience with hydra by six, nine or even twenty-two asexual generations will accept and appropriate nematocysts from hydra upon which they have fed.

Having established the fact that the instinct involved in the manipulation of hydra's nematocysts persists through a score of asexual generations, we next attempted to break the persistence of the instinct by bringing about the intervention of regeneration on the part of *Microstomum* as well as the intervention of asexual generations which lacked experience with hydra.

A specimen was, therefore, taken from the sixteenth asexual generation of clone "A," to be experimented upon, on November 20, 1926. This specimen was held quiet in a shallow layer of water and decapitated along line *a,* Fig. 2. This involved the removal of mouth, pharynx and dorsal ganglia (the only ganglia present in *Microstomum's* nervous system.) There had been no evident fission-plane in this specimen. However, following the cutting, much of the enteron oozed out from the cut end, which was followed by an abstriction along line *c,* Fig. 2. This resulted in two incomplete zoöids *A* and *B*. It is possible that the rudiments of dorsal ganglia might have been present in zoöid *B*. Zoöid *A* was so much reduced because of the exudation of tissues that it was not a promising specimen. It was rejected and zoöid *B* was cared for. Four days later (November 24) zoöid *B* had formed a cephalic region and accepted tadpole liver. After it had fed, a large green hydra was placed with it. Within five minutes three of us, in this laboratory, saw the *Microstomum* feeding upon hydra. On November 26, the specimen displayed many nematocysts within its mesenchyme and a few at the surface. On November 27, many nematocysts were distributed uniformly at the surface of *Microstomum*. Thus it was seen that the posterior zoöid, which was induced to abstrict from the parent prematurely and thus lacked a fully organized complement of anterior organs, accepted and appropriated, after regeneration of an anterior region, the nematocysts of hydra, despite the fact that it had been removed from experience with hydra at least fifteen asexual generations.

We were also prompted to make a similar attempt by using an incomplete anterior zoöid. Accordingly, from the seventeenth generation of the same clone "A," an individual was chosen on November 23 that showed no fission plane when examined under the 4 mm. objective and 10 × eyepiece. The specimen was cut, as it lay in a shallow sheet of water, along the line indicated by *a* in Fig. 3. In reaction to this operation, this specimen, too, divided along the plane indicated by *b* in Fig. 3. The posterior zoöid was rejected. The anterior zoöid had now lost its mouth, pharynx, cephalic ganglia and caudal region. It is possible that near its mid-transverse plane there had been the merest rudiments of cephalic ganglia. It is certain, however, that there were no cephalic ganglia at its anterior wounded end. The recorded history of this zoöid is as follows:

On November 25, two days after the operation, it was not able to swim in a straight course; only after repeated attempts to feed it, was it induced to eat some tadpole liver. On November 27, it was not, as yet, swimming along a straight path. In the meantime, not only had the anterior and posterior structures been fairly well regenerated, but a conspicuous fission-plane had formed in the zoöid's mid-region. The specimen now attempted to feed upon tadpole brain. Its reaction was quite awkward. In trying to assist it, the posterior zoöid was torn from the specimen. This posterior zoöid was discarded. On November 29, the specimen was no longer awkward in its swimming and fed upon tadpole liver. It was then placed with a seven-tentacled green hydra December 4. On December 6, the *Microstomum* had many nematocysts distributed over its surface and the hydra had lost one and part of another of its tentacles. The *Microstomum* then accepted a large meal of tadpole liver. Thus it is seen that the anterior half of a prematurely divided *Microstomum*, from which the mouth, pharynx, and cephalic ganglia had been removed, will accept and appropriate the nematocysts of hydra, even though this partly regenerated zoöid had been removed from experience with hydras by at least sixteen asexual generations.

Meixner ('23) was unable to observe *Microstomum lineare* discharge any of its nematocysts. He further cited the absence of cnidocils or releasing mechanisms associated with the nematocysts of *Microstomum*. He, therefore, challenged the senior author's suggestion that *Microstomum caudatum* uses its nematocysts. Meixner had not seen the records of Kepner and Barker ('24) which were yet in editors' hands. These authors record observations of *Microstomum* discharging at and wounding other animals with its appropriated nematocysts.

Meixner's paper has suggested to us that it might be well to determine whether *Microstomum*, when removed from experience with hydra by ten or more asexual generations, could use the nematocysts that it had appropriated from hydra. Accordingly such specimens were issued to four graduate students.

Mrs. J. S. Carter reports that she "was successful only in so far that I saw them eat hydra and retain the green coloration for at least twenty-four hours, but the *Microstomums* died before I completed the experiment."

Mr. Paul R. Burch also gave a negative report as follows: "A *Microstomum* ten generations removed from the use of hydra as food was received February 28, 1927, and placed with hydra March 2d. The *Microstomum* showed nematocysts the next day, 22.5 hours later. In the meantime it had divided. One of the daughter *Microstoma* was placed in a hanging drop of water in a moist chamber with two *Stenostoma* and observed almost continuously for three hours. During a great part of this time one or the other of the *Stenostoma* was in contact with the *Microstomum* and pushing against it. Time and time again one or the other *Stenostomum* forced its way between the *Microstomum* and a piece of detritus or between *Microstomum* and the other *Stenostomum*, yet the *Microstomum* did not discharge a single nematocyst during this time. It was noticed, however, that at least one third of the nematocysts were not yet oriented for discharge."

Mr. Wesley Fry obtained a *Microstomum,* which was fifteen generations removed from experience with hydra. The animal had no nematocysts. He reported: " I fed this specimen a green hydra and on Saturday, March 5, the animal had appropriated the nematocysts of the hydra. They appeared scattered throughout the ectoderm of the *Microstomum.* I placed this animal in contact with a *Stenostomum* in a hanging drop and saw it discharge nematocysts at the *Stenostomum.*"

Mrs. S. B. Grimes used some *Microstoma* which were fifteen generations removed from contact with hydra and were, therefore, quite free from nematocysts. He reported: " I fed them green hydras and in the course of twenty-four to forty-eight hours the nematocysts became oriented in the ectoderm of the *Microstoma.* I then placed one of these 'loaded' *Microstoma* in a hanging drop along with a small oligochæte and watched the reaction. The setæ of the worm agitated the *Microstomum* so that it oscillated its nematocysts as though to discharge them. The setæ continued to irritate the flatworm and several nematocysts were thrown out. The threads of some of them pierced the body-wall of the oligochæte and partly paralyzed the worm. Dr. B. D. Reynolds and Mr. J. R. Mundie each checked up on what I had seen."

Thus it was demonstrated by two of these observations that a *Microstomum,* removed by as many as fifteen asexual generations from experience with hydra, can not only appropriate the nematocysts of the latter but can actually use these nematocysts and discharge them into the bodies of offending neighbors.

This persistence of factors for instinct of *Microstomum* would seem to be the outcome of the potentialities of the various tissues and cells of the body. For, in the first place, gametes and zygotes have not been involved in our experiments. The persisting of factors for instinct, therefore, could not have resided in the germplasm in this case. Secondly, the neuroplasm has not been involved in transmitting this instinct. Ritter and Congdon ('00) claim that in *Stenostomum* there is a migration of neurones from the anterior zoöid into the incipient posterior zoöid. Child ('02) has not been able to observe such migration. The senior author has made frequent observations upon the histogenesis involved in fission of *Microstomum.* No migration of neurones has been observed by him. On the contrary, the ganglia arise locally as internal epidermal proliferations. The neurones for each generation, therefore, arise independently from the epidermis. Moreover, the conduct of *Microstomum* in manipulating these nematocysts precludes neural control; for the cnidophages carry the nematocysts to the surface as free cells operating quite as independently of the central nervous system's direct control as do the phagocytes of one's own body. And when once they have established themselves at the surface of the *Microstomum's* body they respond to threatening stimuli independently. In this respect they fall under the class of cells that Parker ('19) refers to as "independent effectors" (pp. 83–84). If, on the other hand, the cells attending the nematocysts in *Microstomum* be not cnidophages but cnidoblasts of hydra (as Meixner ('23) maintains), they are quite foreign to the neuroplasm of *Microstomum.*

The persistence of factors for an instinct, independent of the nervous system and so complex as that concerned with the collecting and manipulation of the nematocysts of hydra on the part of *Microstomum* is contrary to the prevailing ideas that the factors for instincts reside in the neuroplasm of the individual and are transmitted through the germplasm. That the factors, which determine the presence of instincts, are considered to lie within the neuroplasma is indicated by two references we have chosen to make. Many others might have been selected. D. Forel, in reviewing the instinctive conduct of ants, calls attention to the fact that the ant's brain surpasses in relative volume and in complication of structure that of all other insects. Again, Charles Darwin ('74) says: "It is certain that there may be extraordinary activity with an extremely small absolute mass of nervous matter; thus the wonderfully diversified instincts, mental powers, and affections of ants are notorious, yet their cerebral ganglia are not so large as the quarter of a small pin's head. Under this point of view, the brain of an ant is one of the most marvelous atoms in the world, perhaps more so than that of man." These two references represent the attitude usually taken concerning the relation between conduct and neural structure.

But in this case, the instinct, involved in handling the appropriated nematocysts, can not be said to be controlled by the central nervous system of *Microstomum*. The control of the manipulation of the nematocysts through hormones is suggested, but it seems better to consider this manipulation to be the outcome of the potentialities of all of the cells of *Microstomum's* body.

There are not many examples on record of factors for an instinct persisting through generations that have not put into operation this instinct. C. R. Griffith ('19) found that white rats, that had been members of the tenth generation of inbreeding, showed a pronounced instinctive fear of a cat, though the previous nine generations had not experienced the presence of a cat. But Griffith says that this reaction on the part of the rats "may not be specifically related to the situation. . . . Any other new stimulus may arouse such reactions, the necessary component of total perception being just the unfamiliarity or strangeness and not the specific feline odor" (p. 167).

In the conduct of *Microstomum*, dealing with the nematocysts of hydra, there is a reaction that is quite definitely specific. Hence we have a clear case of an instinct being transmitted through as many as twenty-two asexual generations though it had not been exercised in twenty-one previous ones.

Summary.

Microstomum caudatum, after being kept from experiences with hydra for twenty-two asexual generations, will feed upon hydra and appropriate the nematocysts. The removal of all of the ganglia and the intervention of extensive regeneration in *M. caudatum*, sixteen asexual generations removed from hydra, will not prevent the transmitting of factors for the instinct that deals with the manipulation of the nematocysts of hydra by *Microstomum*. The appropriated nematocysts have been *used* by an individual as much as fifteen asexual generations removed from hydra.

The instinct is in this case, therefore, not resident in the neuroplasm and the factor determining its presence is transmitted from generation to generation by the soma and not by germplasm.

LITERATURE CITED.

Child.

'02 Studies in Regulation—Fission and Regulation in *Stenostomum.* Arch. Entw. Mech., 15.

Evans, J. T.

'22 *Calma glaucoides:* a Study in Adaption. Q. Jour. Micro. Sci., 66.

Griffith, C. R.

'19 A Possible Case of Instinctive Behavior in the White Rat. Science, 50.

Kepner, Wm. A.

'11 Nematocysts of *Microstomum caudatum.* Biol. Bull., 20.

'11 Concerning the nematocysts of *Microstomum caudatum.* Science, 34.

'25 Animals looking into the future. New York.

Kepner, Wm. A., and John F. Barker.

'24 Nematocysts of *Microstomum.* Biol. Bull., 47.

Martin, C. H.

'08 The Nematocysts of *Turbellaria.* Q. Jour. Micro. Sci., 5.

'14 A Note on the Occurrence of Nematocysts and Similar Structures in the Various Groups of the Animal Kingdom. Biol. Zentralblatt., 34.

Meixner, Josef.

'23 Über die Kleptokniden von *Microstomum lineare* (Müll). Biol. Zentralblatt., 43.

Parker, G. H.

'19 The Elementary Nervous System. Philadelphia.

Ritter, W. E., and E. M. Congdon.

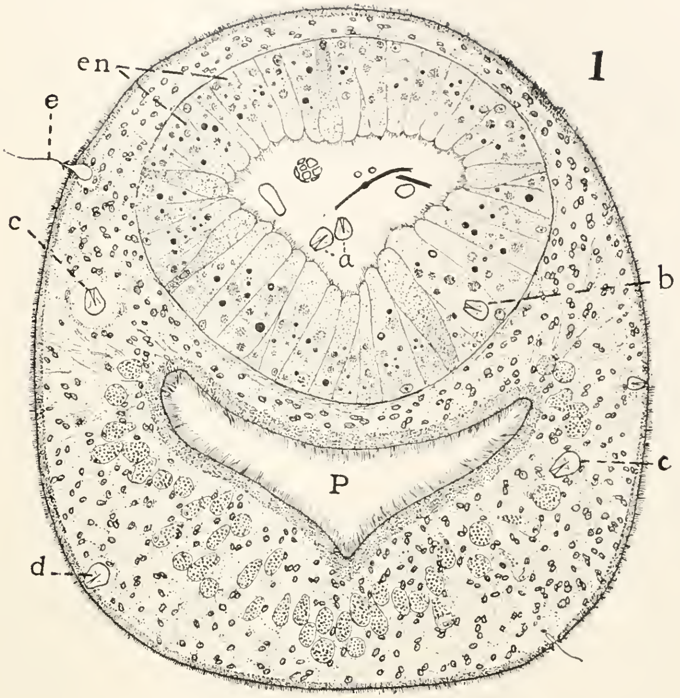
'00 On the Inhibition by Artificial Section of the Normal Fission Plane in *Stenostoma.* Proc. Calif. Acad. Sciences, 3d ser., Zoölogy, Vol. 2, No. 6.

EXPLANATION OF FIGURES.

FIG. 1. Transverse section of *M. caudatum* taken through plane shown by *m* in Fig. 2. *a,* naked nematocysts of hydra lying within lumen of enteron; *b,* naked nematocyst within a vacuole of an endodermal cell; *c* and *c,* naked nematocysts within lacunæ of mesenchyme; *d,* nematocyst oriented and accompanied by a cnidophage, the nucleus of which is shown at base of nematocyst; *e,* partly discharged nematocyst; *en,* endoderm of enteron; *P,* pharynx. From Kepner ('25).

FIG. 2. Specimen fifteen asexual generations removed from experience with hydra. *a,* plane along which body was severed; *c,* plane along which body abstricted after the anterior end had been cut away, thus forming two incomplete but free zoöids (*A* and *B*) ; *m,* plane of transverse section shown in Fig. 1.

FIG. 3. Specimen sixteen asexual generations removed from hydra. *a,* plane along which specimen was severed; *b,* plane along which body abstricted after the anterior end had been cut away, thus forming two incomplete but free zoöids (*A* and *B*).



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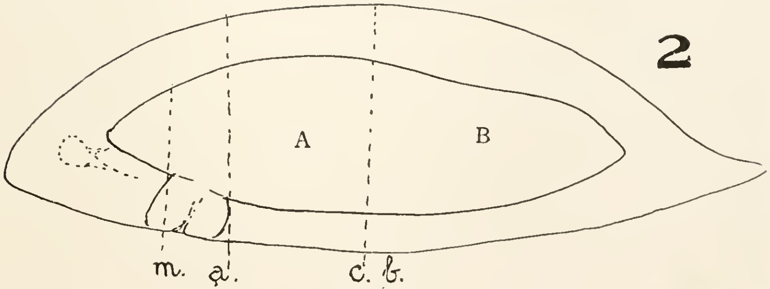

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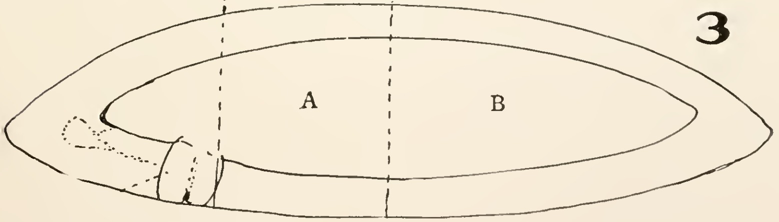

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W. A. KEPNER AND J. W. NUTTYCOMBE.

STUDIES ON THE PHYSIOLOGY OF THE EUGLENOID FLAGELLATES. I. THE RELATION OF THE DENSITY OF POPULATION TO THE GROWTH RATE OF *EUGLENA.*

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Introduction.

The relation of volume of culture medium to the rate of growth of ciliates has been investigated by a number of workers during the last two decades, but up to the time of this investigation no similar experiments on the green flagellates had been attempted. The object of the present investigation, therefore, was to determine whether or not such a relationship exists in growth of *Euglena,* and also whether or not there is evidence for an "allelocatalytic effect," such as described by Robertson (1921*b,* 1922) for ciliates.

For this series of experiments *Euglena* sp. was chosen for its ability to grow rapidly in "autotrophic" media. Also, in such a medium the food supply of an organism is much less affected by bacterial activities than if a large amount of organic material were present. The first experiments were carried out in the same manner as were previous isolation experiments with ciliates (Woodruff, 1911, 1913; Robertson, 1921*a, b,* 1922; Cutler and Crump, 1923*a, b,* 1924, 1925; Greenleaf, 1926; Myers, 1927; Yocom, 1928). This method was discarded later in favor of a mass method in which variations in the concentration of organisms were substituted for variations in volume of the medium.

This investigation was carried out at the suggestion of Doctor R. P. Hall, and the writer wishes to express his gratitude for suggestions and aid in the preparation of the present paper. The writer also wishes to thank Professor Asa C. Chandler for the use of the Biological Laboratory of the Rice Institute during the summer of 1928.

HISTORICAL SURVEY.

The earliest observations on the relation of volume of culture medium and division rate were those of Woodruff (1911) on two species of *Paramecium* (*P. caudatum* and *P. aurelia*), which showed a more rapid division rate in larger than in smaller volumes of the same culture medium. Woodruff suggested that this was due to a more rapid accumulation of waste products in cultures of smaller volume, and in later observations (Woodruff, 1913) he showed that waste products of any one species (of *Paramecium, Stylonychia, Pleurotricha*) actually depressed the division rate of the same species, although apparently without effect on other species.

The next investigations in this field were carried out by Robertson (1921*a, b,* 1922, 1924*b, c*) who found that when two ciliates were isolated into the same drop of culture medium the division rate was higher (sometimes sixteen times as great) than if a single cell were isolated into a drop of the same size. This difference in division rate is, according to Robertson, due to the mutual contiguity of the cells, and has been designated by him as an "allelocatalytic effect," caused by a growth-catalysing substance liberated from the nucleus into the cytoplasm and thence into the surrounding medium during nuclear division. This theoretical catalyst is called by Robertson an "autocatalyst of growth." The amount of this catalyst present in isolation cultures would depend upon the number of cell divisions that had taken place; therefore, the culture with the greater number of dividing cells would have a greater concentration of catalyst, and the division rate in these cultures should be correspondingly greater.

Cutler and Crump (1923*a, b*) found no evidence for the occurrence of allelocatalysis in cultures of *Colpidium* with 1, 2, 3, and 4 initial organisms in volumes that varied from 0.5 to 8.5 cubic millimeters, or in mass cultures with concentrations of 100–8,000 organisms per cc. They pointed out that their results as well as those of Robertson, might be explained by the presence of a larger food supply in the cultures showing a higher division rate.

Greenleaf (1924, 1926), in a large number of experiments in which the volume of medium was varied from two to forty drops, found that two species of *Paramecium* (*P. aurelia, P. caudatum*) and *Pleurotricha lanceolata* showed a higher division rate in larger volumes of medium, and also that in cultures of *Stylonychia pustulata* the division rate of a single animal was greater than that of either of a pair of animals when introduced into the same volume of medium.

Calkins (1926) made a sixty-day test of *Uroleptus mobilis* with one, two, three, and four individuals per drop. The cultures with one initial organism showed a higher division rate than those with two organisms, and those with two organisms a higher division rate than those with three and four.

Myers (1927), in a long series of experiments, found that the division rate of *Paramecium* decreased with the density of population, and that a certain minimum volume (0.2–0.4 cc., or 4–8 drops) of "flourishing" hay infusion was necessary for a single *Paramecium* to multiply at its maximum rate.

Yocom (1928) found that in *Oxytricha* four-drop cultures had a 14 per cent. higher division rate than ten-drop cultures. In each series of experiments, however, there were some cases in which the division rates of the ten-drop cultures were equal to or higher than those of the four-drop cultures. Only by averaging a number of cases could he show an appreciable difference. He accredits this difference to the earlier attainment of a high concentration of autocatalyst in the cultures of smaller volume.

Petersen (1929) has found that within certain limits a higher division rate of *Paramecium* is present in cultures of smaller volume and in cultures containing a larger number of ciliates. Some of her results, however, tend to contradict Robertson's theory.

In reviewing the work on volume-division rate relations in Protozoa one finds that Robertson's theory is supported only by the work of Yocom and by some of the results of Petersen. On the other hand, the experiments of Woodruff, Cutler and Crump, Greenleaf, Calkins, Myers, and some of the results of Petersen, furnish evidence which contradicts the theory of an autocatalyst.

Material and Methods.

Early in the summer of 1928 water was collected from several ponds in the vicinity of Houston, Texas, including that known as the "Biology Pond" of the Rice Institute. This was placed in aquarium-jars and gave rise to mixed euglenoid cultures. An autotrophic medium similar to those used by Mainx (1928) and Günther (1928) was prepared as follows:

NH_4NO_3	10 gms.
KH_2PO_4	5 gms.
$MgSO_4$	1 gm.
H_2O (distilled)	1000 cc.

This stock solution was diluted ten times, brought to pH 7.0 with Na_2Co_3, and then boiled before being used. Culture material was centrifuged, the supernatant liquid poured off, and the synthetic medium added. By repeated centrifugings and washings over several days the organic content was reduced to a minimum. Some of the *Euglena* present continued to grow and gave rise to a mass culture in the autotophic medium. By means of a mouth-controlled suction pipette single specimens of *Euglena* sp. were isolated into fresh medium in depression slides. All *Euglena* used in subsequent experiments were descendants of a single specimen isolated at this time. The depression slides were kept on racks in dessicators and large petri dishes half filled with water.

In order to determine the relation, if any, of volume of culture medium to division rate, sister cells were isolated into different amounts of medium. On account of the small size of the flagellates, the most practicable volumes for such cultures ranged from two to six drops. In all, twenty-two pairs were started at various times. In every case a flagellate was chosen at random from the mass culture derived from a single *Euglena*. Each organism was isolated into a depression slide. After the first division the daughter cells were washed twice in three drops of medium, then placed in separate depressions on another slide and immediately covered with fresh medium—one with two drops, the other with six. For each pair of cultures, counts were made daily and the numbers present recorded.

At the end of ten days, three pairs of cultures showed from two to three and a half times as many organisms in the cultures of smaller volume. In one pair, however, three times as many were present in the culture of larger volume. In another instance the numbers were about equal. The rest of the cultures were discarded on account of excessive evaporation. Such results are by no means conclusive, since they indicate a marked variability in the division rate of this flagellate.

The isolation method in this case, therefore, seemed to be unreliable, and was abandoned for the following reasons: (1) The mortality rate was high, due to the evaporation of the medium. (2) Evaporation increased the salt concentration, and consequently the osmotic pressure of the medium. (3) The practicable range of variation in volume was limited (two to six drops). (4) The results obtained were contradictory. In many cases, synchronous division did not occur in pure lines derived from sister cells left undisturbed in the same depression slide. (5) An extremely large number of experiments would be necessary in order to obtain growth curves that would approach accuracy. (6) Bacterial counts could not be made for such cultures without greatly altering the volume of the medium. In order to avoid as many as possible of these difficulties the mass culture method was adopted.

The same pure line used in isolation cultures was grown in mass cultures in the autotrophic medium already described. By repeated centrifuging and washing, cultures with low bacterial counts were obtained. These were concentrated in centrifuge tubes, and various dilutions were made. In 18 × 40 mm. test tubes, 20 cc. of each dilution was kept at room temperature under a constant source of light, consisting of a battery of three 100-watt globes (frosted on the inside). In series I., II., III., and IV., the cultures were protected from the heat by a wind tunnel with top and bottom of thin plate glass. An eight-inch fan removed practically all of the surplus heat. The battery of lights was above the tunnel and the cultures below. Mirrors were placed both above the lights and below the cultures in order to insure as uniform a distribution of light as possible. The number of organisms per cc. was counted at the beginning of the experiment and at convenient intervals thereafter. Bacterial counts were made by plating according to the standard method used in water analysis.

Bacterial counts were made on each dilution at the beginning and at the end of each series and in some cases at the end of thirty-six hours also. Initial counts were always low, ranging from 200 to 1,500, and in a few cases to 3,000 per cc. Thirty-six hour counts were much higher, ranging from 20,000 to 50,000 per cc. Final counts ranged from 30,000 to several hundred thousand per cc. Since the effect of bacterial products on the rate of division of *Euglena* is unknown in this case the significance of the counts can not be stated. It is worth noting, however, that the larger number of bacteria after the first day did not result in an increased division rate. On the contrary, the division rate in cultures of *Euglena* sp. decreased continuously after the first few divisions, thus indicating that their growth was either hindered or else unaffected by the increase in number of bacteria. If bacteria hindered the division rate, this might possibly be effected in one or both of two ways—by utilizing and thereby decreasing the food supply of the euglenoids, or by excreting products of an injurious nature into the medium. However, there is no evidence supporting either possibility, or indicating that the bacteria affected the division rate of the flagellates in any way.

In series V. and VI., Carrel tissue culture flasks were used instead of test tubes. The Carrel flasks assured a much more uniform distribution of light than did the test tubes. The flasks were only half filled in order to allow air circulation. They were suspended in a wire rack and partially immersed in a Freas Water Thermostat (of the small type, accurate to .1° C. for long periods of time) at 27° C.

A Sedgwick-Rafter counting chamber of 0.5 cc. capacity and a Whipple micrometer were used for counting. The method of counting was, in general, that described by Whipple (1927) for counting plankton. Each tube or flask was sampled twice, and usually seventy-two 1 mm. squares were counted and averaged for each sample. If the counts for the two samples differed as much as ten per cent., a third count was made. These three counts were then averaged, and the result was taken as the count for the tube or flask. When duplicate flasks were used each flask was sampled twice, and a count was made for each. The counts for the two flasks were then averaged to find the final count for the dilution in question. In determining the initial concentrations, the average count of five samples was taken in order to insure as great accuracy as practicable. Pipettes for filling the counting chamber were washed thoroughly, autoclaved or boiled, and then dried before being used.

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In all cases the concentration of flagellates has been expressed as thousands per cc. or decimal fractions thereof. For example, a concentration of 540 individuals per cc. is expressed as .54 thousand per cc., or merely as .54. No large series of counts was undertaken to determine the exact percentage error, but it is believed that the error is rather low. For example, one set of initial counts was as follows: .87, .90, .87, .91, .92, with an average of .894. Assuming that the flasks were well shaken before sampling, the standard error of such a system of counting is plus or minus the square root of the total number counted (Fisher, 1925, p. 59). In the set of initial counts mentioned above this would be $\sqrt{321}$ or ± 18, giving as the total corrected number 321 ± 18, or a concentration of .894 ± .047. The probable error is always .67499 times the standard error, and in this case is $.67499 \times \pm .047$, or ± .0317, making the concentration .894 ± .032. This is a probable error of 3.52%. However, since the greatest deviation of the individual counts from the mean is .026 in the series mentioned, it is fairly certain that the error is very much less than the standard and is well within the probable error. Concentrations of less than .5 were not counted because of the large number of squares that would have to be counted in order to insure a fair degree of accuracy.

It may seem that four hundred organisms per cubic centimeter is a high concentration, and that from the beginning the cultures might suffer from crowding. However, if the total volume of the organisms is compared to the volume of the medium, it is seen that this is not the case. Taking the radius of a rounded organism as approximately eleven microns, we may calculate its volume to be 4.189×11^3, or approximately 5,575 cubic microns. Since one cubic centimeter is equal to 10^{12} cubic microns, the ratio of the volume of 450 *Euglena* to that of their containing fluid is $\frac{450 \times 5.575}{10^{12}}$, $\frac{2,608,750}{10^{12}}$, or approximately 1 : 383,300. This means that in the most concentrated cultures of all except the first series of experiments, the initial volume of the flagellates was approximately 1/383,300 that of their surrounding medium, while in the most dilute cultures the volume ratio of organism to medium was 1 : 3,833,000.

EXPERIMENTAL RESULTS.

Series I.

On October 14 the organisms were washed and centrifuged six times. On October 15 they were centrifuged five times before the experiment was started. Since the centrifuge tubes were filled to approximately 18 cc. and about 17.75 cc. of supernatant liquid was poured off each time, the concentration of any catalyst of growth, if present, was reduced to approximately 1/72 $\left(\frac{18 - 17.75}{18}\right)$ of its previous concentration with each washing. Two consecutive washings would reduce the autocatalyst to approximately 1/5,000 of its original concentration; five washings to 1/1,900,000,000. The organisms, after washing, were concentrated by centrifuging and then counted. The concentration was found to be 39.2 thousand per cc. Various numbers of organisms were placed in five culture tubes, which were then filled with medium to a volume of 20 cc. The concentration was then determined for each tube. The initial count in tube number 1 was 0.42; in tube 2, 0.82; tube 3, 1.9; tube 4, 5.0; tube 5, 9.5.

Counts were made subsequently at intervals of 24–36 hours. The average counts were accurately plotted against time, as shown in figure 1. In Fig. 2 the same curves are repeated, but in this case x/x_0 (where x represents the initial number, *e.g.*, .42 in tube number 1) is plotted against time; in other words the curves shown in Fig. 1 are all reduced to unity in Fig. 2. It is readily seen that, in general, the increase in number per initial organism was much greater in the more diluted cultures (Fig. 2). An objection to the concentration used is readily seen in the fact that the more concentrated cultures were initially quite crowded; hence they were probably restricted sooner and more severely by limiting factors than were the more diluted ones. Therefore, other series of experiments were started in which all dilutions were higher than that of culture 1 of series I. It was hoped that these would yield a set of curves in which the limiting factors would be negligible for the first few days. The results of such experiments are described in series II., III., IV., V., and VI.

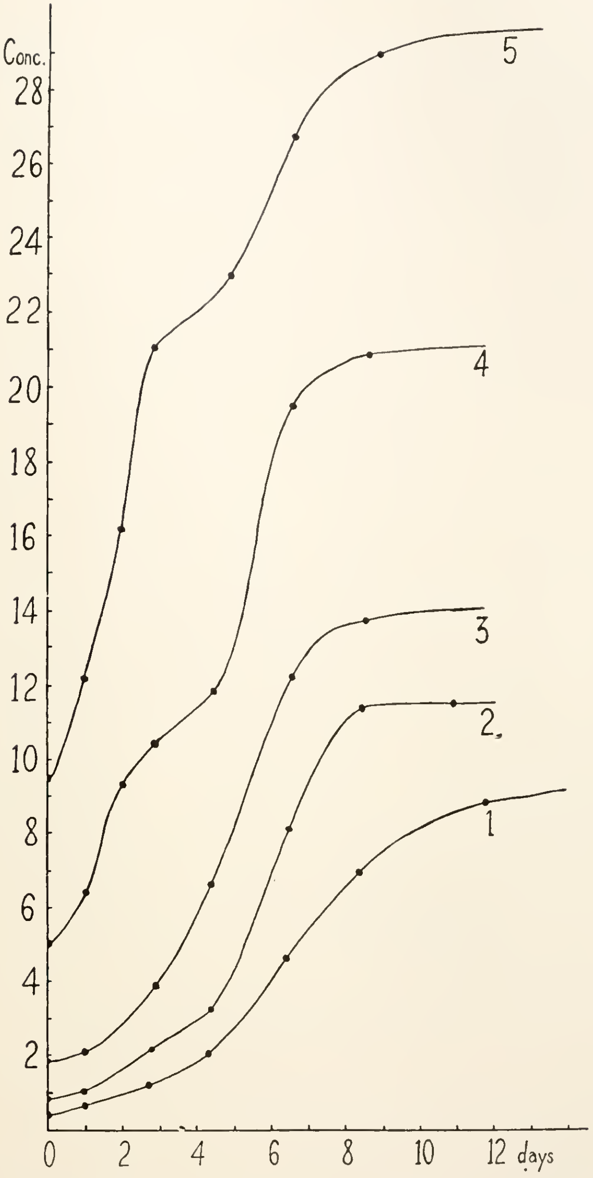

Fig. 1. Graph showing concentration of organisms (in thousands per cc.) plotted against time for series I. Curves 1, 2, 3, etc., represent the numbers present in tubes 1, 2, 3, etc., respectively. Dots represent observed points.

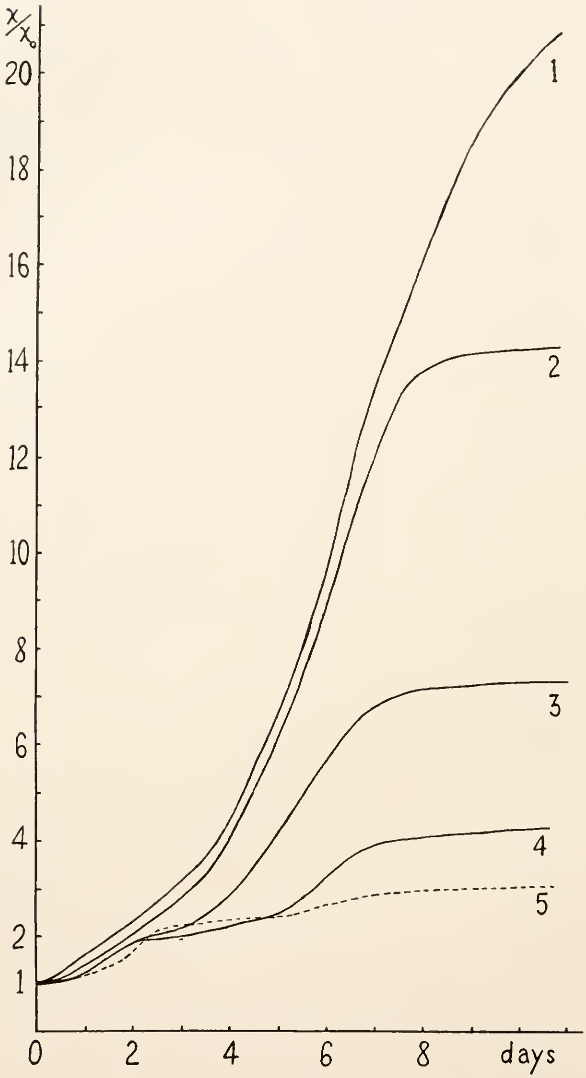

FIG. 2. Graph showing x/x_0 (x_0 being the number at any time t; x_0, the initial number) of the curves of Fig. 1 plotted against time. Note that the order of the curves is reversed. The most concentrated culture (curve 5) shows the smallest increase (with a ratio of 3 : 1), while the most dilute (curve 1) shows the largest increase (ratio of 21 : 1). The others show intermediate ratios varying in reverse order with their original concentrations. These curves thus indicate higher rates of division in the less concentrated cultures.

Series II.

The organisms were centrifuged a number of times over several days, and three times just before the experiment was started. Three dilutions were used, and they were maintained in duplicate, the tubes being designated as 1*A*, 1*B*, etc., respectively. One concentration was made and counted. It was found to be .555. Twenty cc. of this was placed in tubes 3*A* and 3*B*. Part of the remainder was diluted one to three with sterile medium to make a concentration of .14. Twenty cc. of this was placed in tubes 2*A*

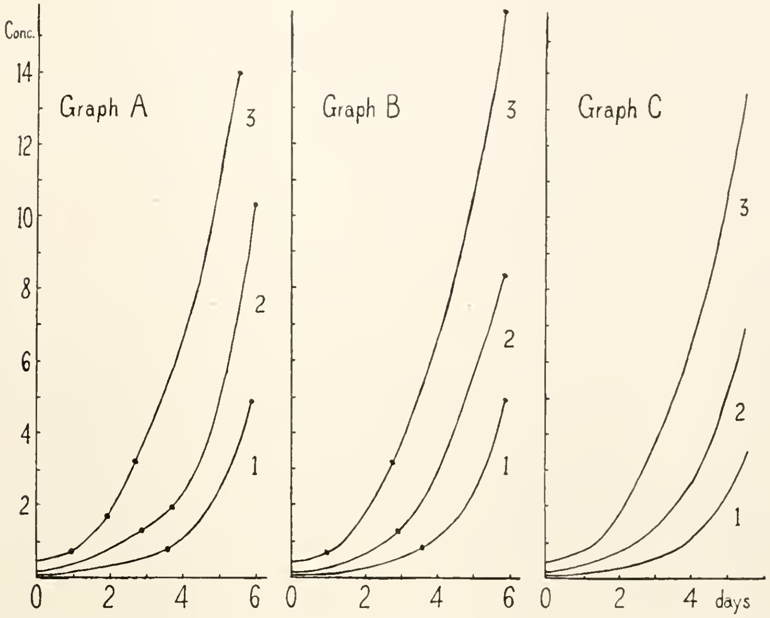

FIG. 3. Graphs showing concentrations for series II. plotted against time.
Graph *A*.—Curves 1, 2, and 3 show numbers in cultures 1*A*, 2*A*, and 3*A*, respectively. Dots indicate observed points.
Graph *B*—Curves 1, 2, and 3 show numbers in cultures 1*B*, 2*B*, and 3*B*, respectively. Dots indicate observed points.
Graph *C*—A combination of graphs *A* and *B*, obtained by averaging corresponding values for the curve of the two preceding graphs.

The fact that graphs *A* and *B* are very similar and that the values of x for any dilution at any given time do not vary much in the two sets of cultures is a demonstration of the efficiency of the method used. Initial numbers were the same for the corresponding curves, conditions were the same for the two sets of cultures, and the resulting curves are very similar.

and $2B$. The remainder was diluted one to nine to make a concentration of .055, of which 20 cc. was placed in tubes $1A$ and $1B$. The results are shown in Fig. 3. Laboratory conditions prohibited the continuance of the experiment more than six days. Fig. 4 shows x/x_0 of the preceding curve (Fig. 3) plotted against time. The higher division rates of the more dilute cultures are obvious, especially after the first few days when the accumulative differences become quite large.

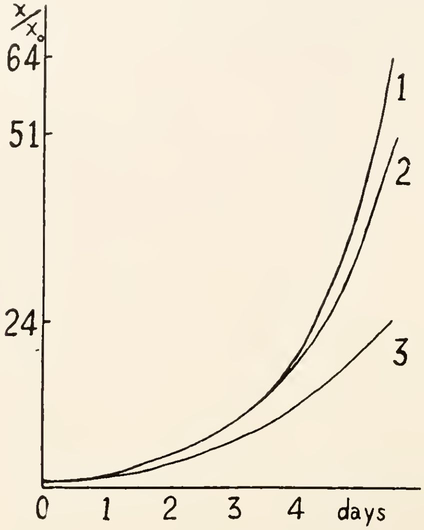

FIG. 4. Graph showing x/x_0 (x being the number at any time t; x_0, the initial number) of graph C, Fig. 3, plotted against time. Note the higher relative rate of increase (ratio 64 : 1) in the more dilute culture represented by curve 1, as compared with the most concentrated culture represented by curve 3 (ratio 24 : 1). This is directly opposed to any theory of an autocatalyst.

Series III.

The organisms were centrifuged at least a dozen times during several days and three times just before the beginning of the series. The flagellates were counted. The concentration in tubes $3A$ and $3B$ was .35. The dilution was 1 to 3 for tubes $2A$ and $2B$ and 1 to 9 for tubes $1A$ and $1B$, as in the previous series. At the end of the first 24 hours (Fig. 5) the count for the most concentrated cultures was .73, and at the end of 48 hours was 1.32, and at the end of 72 hours was 2.07 as shown in Fig. 5. At the end of 72 hours the count for the second dilution (tubes 2*A* and 2*B*) was .51. Due to an accident no count for the highest dilution could be obtained. For this reason the series was discontinued. The initial concentration in tubes 2*A* and 2*B* was ¼ of that in tubes 3*A* and 3*B*. This ratio was still maintained at the end of the third day when counts were .51 and 2.07.

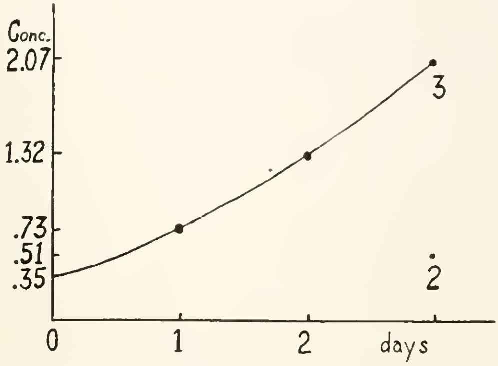

FIG. 5. Graphs showing concentrations in series III. plotted against time. Due to an accident data for one curve and for only one point on another curve was obtained. These were dilutions 3 and 2. The ratio of the initial concentrations was 4 : 1, and the ratio of their final concentrations at the end of the third day was 2.07 : .51—practically the same as the original. This experiment shows no difference in division rate in the two sets of cultures and is opposed to the theory of an autocatalyst.

Series IV.

The flagellates were centrifuged several times, dilutions were made exactly as in series II. and III., and cultures were maintained in duplicate. The initial count for the most concentrated cultures (dilution 3) was .894. The concentration of dilution 2 was .233; that of dilution 1 was .089. The final counts at the end of seven days on the three sets of cultures (six tubes) showed no large difference of growth rate. The final counts were 0.61, 1.50, and 6.68 for dilutions 1, 2, and 3 respectively; whereas their initial ratio was 1 : 2, 5 : 10.

From this it can be seen that the cultures have maintained approximately their original ratio of concentrations. A slight gain is seen in the most concentrated (dilution 3) over the more dilute cultures (dilutions 1 and 2). Dilutions 1 and 2, however, show the same ratio at the end as at the beginning of the experiment. The conclusion to be drawn from this series is that the division rate in the more concentrated cultures is at least equal to or perhaps slightly greater than that in the more dilute cultures.

Series V.

The organisms were washed at least a dozen times over a period of several days and three times just before the experiment was started. Dilutions were made exactly as in the previous series, the

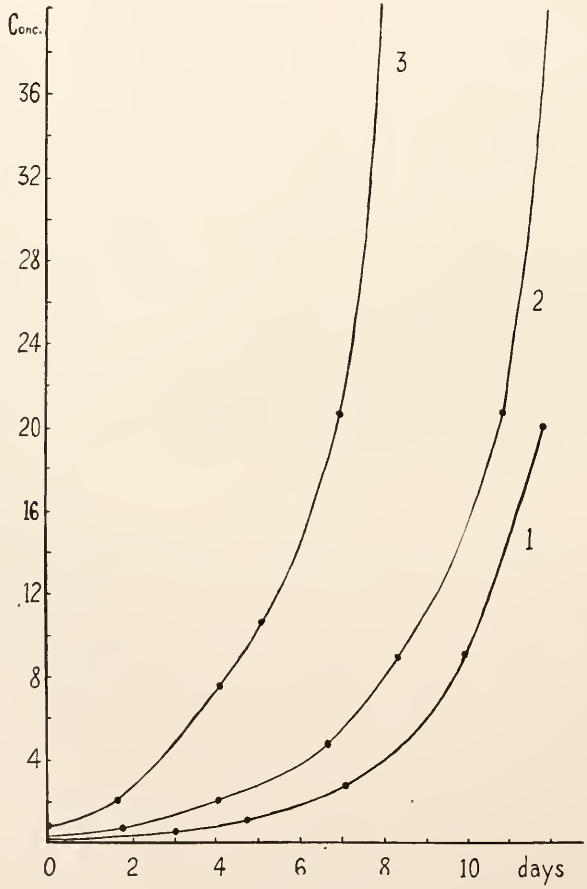

FIG. 6. Graph showing the concentrations of series V. plotted against time.

initial concentrations being .066, .165, and .66 for dilutions 1, 2, and 3 respectively. Duplicate cultures were maintained as in the previous series. The results obtained are shown in Fig. 6, and the curves are reduced to unity in Fig. 7. The increase in numbers was proportionately higher in the more dilute cultures.

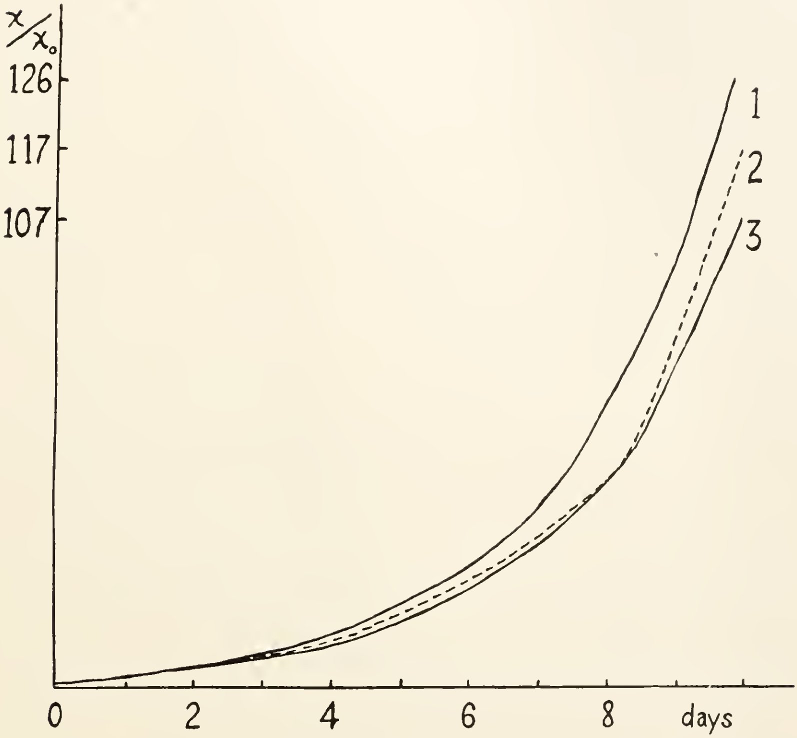

FIG. 7. Graph showing x/x_0 (x being the number present at any time, t; x_0 the initial number) of Fig. 6 (series V.) plotted against time. Note that the order of the curves is reversed from that shown in Fig. 6, denoting a greater relative increase in the most dilute culture (curve 1) at the end of the tenth day (ratio of 126 : 1) than in the most concentrated culture (curve 3, with a ratio of 107 : 1). This is directly opposed to the theory of an autocatalyst.

Series VI.

The organisms were washed, and dilutions were made as in previous series. Initial concentrations were .055, .14, and .55 in dilutions 1, 2, and 3 respectively. These were maintained in duplicate. The results are shown in Fig. 8, and the curves are reduced to unity in Fig. 9. The rate of increase in number was proportionately the same in the lowest and in the highest dilutions for the first seven days. Then the most dilute showed a larger increase than the most concentrated. The intermediate dilution (dilution 2) showed a higher increase on the sixth, seventh, and eighth days, a lower increase on the ninth and tenth days, and a higher increase on the eleventh day than the other dilutions. The reason for this is not known. Since no large differences were

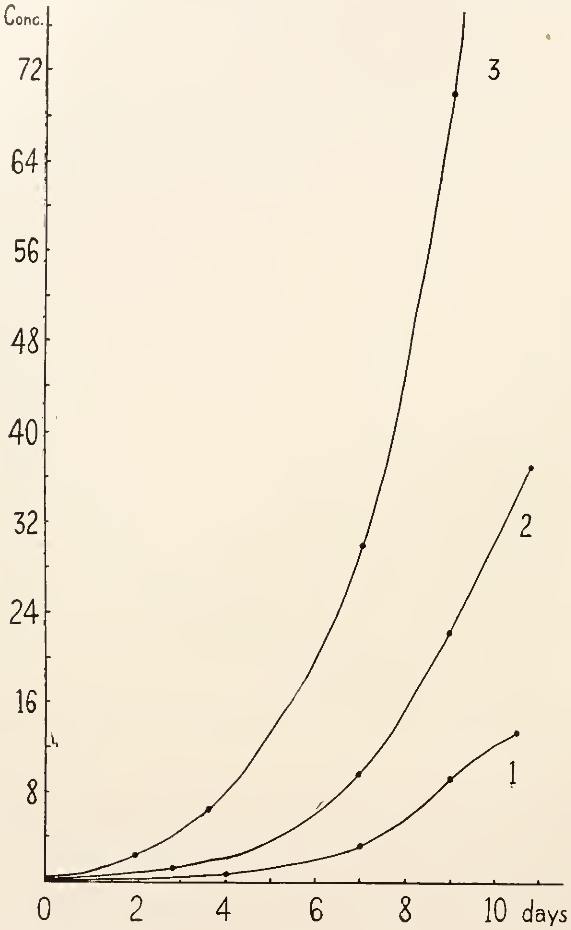

FIG. 8. Graph showing the concentrations in series VI. plotted against time.

observed in the relative amount of increase and since the minor differences were contradictory, the only conclusion to be drawn is that the concentration of organisms had no significant effect on the division rate.

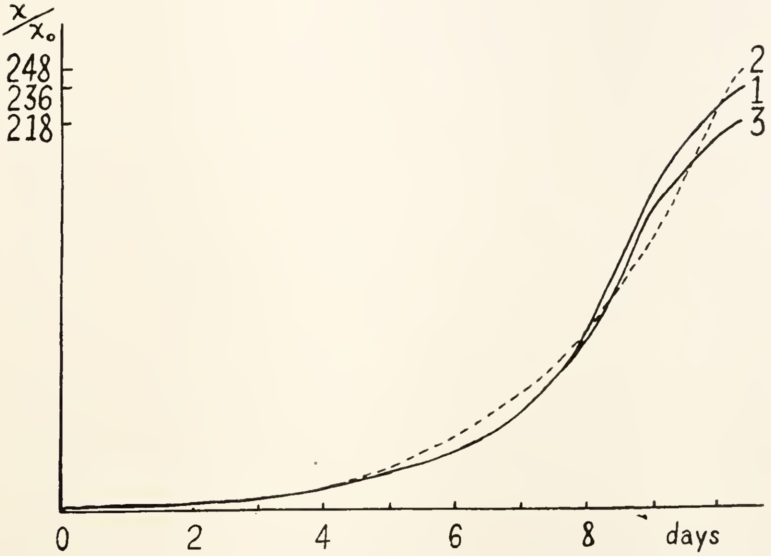

FIG. 9. Graph showing x/x_0 (x being the number present at any time t; x_0, the initial number) of the curves in Fig. 8 (series VI.) plotted against time. A difference in relative amount of increase becomes apparent between the lowest and highest dilutions after seven and one half days, the final ratios being 236 : 1 for the highest dilution (curve 1) and 218 : 1 for the lowest dilution (curve 3). The relative increase of the intermediate dilution is somewhat erratic, being higher during the sixth, seventh, and eighth days, lower during the ninth and tenth, and higher on the eleventh. These results are, of course, contradictory and indicate only that there is no large difference in division rate between cultures of low and high dilution.

DISCUSSION.

As a whole, the results of the writer's experiments with *Euglena* offer no evidence of any allelocatalytic effect. In most of the cultures, especially after the first few days, a significant difference in the opposite direction was apparent. This may be easily explained on the basis of food supply, for the more concentrated cultures would certainly use more food than the more dilute ones, leaving a lower concentration of foodstuffs in the surrounding medium and thus directly depressing the division rate. The more concentrated cultures would also contain a higher concentration of waste products. The most probable explanation of a higher division rate in the more dilute and a lower rate in the more concentrated cultures is that the flagellates in the higher concentrations were hindered by a scant supply of food or a relatively large amount of waste products, or more likely by both these factors.

This conclusion is in accord with the experiments of Woodruff (1911, 1913) who found that waste products of one species of ciliate inhibited the division rate of the same species; and also with the observations of Greenleaf (1924, 1926) that in several ciliates a higher division rate occurred in larger volumes of medium, and that no allelocatalytic effect was present. These results are also in agreement with those of Cutler and Crump (1923*a, b,* 1924, 1925) for both isolation and mass cultures of *Colpidium.* Cutler and Crump (1924, 1925) explained all their results as being due to differences in food supply between large and small volumes, and their data is directly opposed to any theory of an autocatalyst.

Petersen (1929) points out that some of Cutler and Crump's (1923*a*) results indicate a statistical difference in division rate which is in favor of an allelocatalytic effect—a fact which is admitted by the authors. However, this is true only in a limited range of volume (0.5–2.5 cubic millimeters) and only when their one-animal cultures are compared to their two-, three-, and four-animal cultures. If the theory of allelocatalysis is applicable, one should expect to find differences between two- and three-, and between three- and four-animal cultures, and a very marked drop in division rate when single individuals were isolated into very large volumes. Cutler and Crump's (1923*a, b*) results are exactly opposite to this. If their data for two-, three-, and four-animal cultures in 2.5 to 8.5 cubic millimeters are considered, a higher division rate is obvious in the larger volumes within this range. If all volumes from .5 to 8.5 cubic millimeters are considered, however, the average division rates are about the same, regardless of whether the initial number of ciliates was one, two, three, or four. This indicates a total absence of any allelocatalytic effect. It is difficult to understand why Petersen should draw her conclusions only from Cutler and Crump's small-volume cultures, when data from their other volume variations are directly contradictory.

Furthermore, in a paper which Petersen has apparently overlooked, Cutler and Crump (1924) have demonstrated a linear relationship between the average division rate and the number of initial organisms when the initial numbers are plotted in geometrical progression. In other words, they found a positive correlation between volume of medium and division rate, whereas a negative correlation between volume and division rate would be necessary to support Robertson. This relationship is shown in both isolation and mass cultures. In Cutler and Crump's earlier paper (1923*b*) this correlation was masked by the fact that the initial numbers were not plotted in geometrical progression but were given in tabular form. Even when plotted in arithmetical series they show a correlation coefficient of — .37 between division rate and initial concentration of organisms in mass cultures. This, of course, would correspond to a positive correlation of division rate and volume of medium per organism. Although this is a low correlation coefficient, it is significant because it exceeds its probable error by eight and a half times, and any correlation coefficient which exceeds its probable error may be deemed significant. In view of this overwhelming evidence it is certainly impossible to accept Petersen's (1929) interpretation of Cutler and Crump's (1923*a, b,* 1925) results. Their own conclusion (1924, 1925), that the division rate is higher in cultures with a lower concentration of organisms, is well founded and supported by their experiments, and is directly opposed to any theory of an autocatalyst.

Myers (1927 found that the division rate of *Paramecium* decreased with the density of population. Peterson (1929), however, shows that by selecting certain data from Myers' paper she could find some evidence for allelocatalysis. Myers, by averaging five lines, computed the mean generation time to the first division for 1, 2, 4, and 8 individuals isolated into 2, 4, 8, and 16 drops of medium. He performed three series of experiments—one series each with fresh, one-day-old, and two-day-old medium. In most cases the time to the first fission increased with the density of population. The two sets of experiments selected by Petersen are the sixteen drop cultures from the one- and two-day infusions, although the ten other similar sets of experiments show more definite and exactly opposite results. These two sets, however, do show a difference of 2.4 hours in time to the first fission between one- and eight-animal cultures. Myers recorded the number present at the end of six, twelve and twenty-four hours in each of the five lines, thus determining the generation time of each line as six, twelve, or twenty-four hours. The mean generation time was then obtained by averaging the five lines. It is very unlikely, however, that these mean generation times are accurate because the intervals between counts (six hours) were approximately equal to the average generation time. Therefore it was impossible to obtain mean generation times that were accurate within two or three hours, especially when only five lines were considered. For example, if an organism had divided at the end of six and one half hours after isolation, the division would not have been counted until the end of the second six-hour period, and its generation time would go on record as twelve hours—an error of almost six hours or almost 100 per cent. of its true generation time of six and one half hours. In view of the small number of organisms involved, the time between observations in such isolation experiments should not be more than a fraction of the generation time of the organisms under observation, and should be much less than the expected difference which is to be measured. Since, in this case, the periods between observations were almost equal to the generation time, the value of the mean is accurate only within two or three hours, which is 33–50 per cent. of its probable true value. Therefore it is very unlikely that the 40 per cent. difference (2.4 hours) which Petersen calculated between one- and four-animal cultures has any significance at all except perhaps to show that the data which she chose from Myers' paper were entirely insufficient for such an analysis. It is also very difficult to understand how she could consider these two 16-drop series more important than the other ten sets which were performed with the same material under similar conditions, and yet gave opposite results. It is evident, therefore, that the only results of Myers (1927) which seem to indicate an allelocatalytic effect are not significant and that the interpretation placed upon these experiments by Peterson (1929) can not be valid. In view of his results, Myers' own conclusions are logical, are supported by his evidence, and are opposed to the autocatalytic theory.

Robertson (1921*b*), in a series of experiments in which he isolated single ciliates and pairs of ciliates, chosen at random from young parent cultures, into the same volume of medium, found that, instead of twice as many organisms in the two-cell as in the single-cell cultures, there were from three to six times as many at the end of his observations. In isolations made from parent cultures over three days old, there were 2 to 2.7 as many in the two-cell cultures. Since all initial cells were isolated at random from mass cultures, the time since the last division was unknown, and the differences in the ages of the isolated cells might have been almost as much as the generation time of the organism. It can be shown also that the differences obtained by Robertson are not significant and are easily interpreted on a basis other than allelocatalysis.

Cutler and Crump (1924) found that cultures of *Colpidium* with few bacteria showed low division rates and that the organisms showed definite signs of hunger, while cultures with numerous bacteria showed a very high division rate. Other workers have tried unsuccessfully to obtain bacteria-free ciliates which would continue to reproduce. Peters (1921) believed that he was successful, but Cutler and Crump (1924) showed that his cultures were probably contaminated with a small bacillus. Parpart (1928) was successful in freeing *Paramecium* of bacteria, as were also Hargitt and Fray (1917) and Phillips (1922). Parpart makes no mention whatsoever of division in such " sterile " cultures, although he states that the organisms lived as long as five days. The only possible conclusion is that *Paramecium* does not reproduce in sterile culture medium. Hargitt and Fray (1917) and Phillips (1922) freed specimens of *Paramecium* from bacteria and then fed them known pure cultures of bacteria. Since the free-living ciliates have not been known to live without bacteria, and since there is a definite relationship between the number of bacteria and the division rate of ciliates, it would seem probable that the slow multiplication in Robertson's (1921*b*) one-cell cultures was due to lack of food in the form of bacteria.

The difference in growth rate between one- and two-animal cultures shown in Robertson's experiments is not significant because variations obtained in his one-cell cultures are greater than the differences which are accorded to allelocatalysis. Some of his experiments, performed under supposedly identical conditions, are reanalyzed below in Table I.; since he quotes them in several publications (1921*a*, 1923, 1924*a*) they may be accepted as typical experiments. The data for the second and third columns of the table were taken from Robertson's graph.

TABLE I.

Culture Number.	Number present at First Observation.	Hours to First Observation.	Generations.	Average Generation Time to First Observation (in Hours).
238*A*	13	22	3½ approx.	6.3
240*A*	8	23	3	7.7
237*A*	8	22	3	7.3
242*A*	4	24	2	12.0

These generation times of various one-animal cultures of the *A* series show a variation of 5.7 hours, *e.g.*, culture 238*A* had an average generation time of 6.3 hours, and culture 242*A* a generation time of 12 hours. This is a variation of ± 31 per cent. from the median of 9.1 hours in cultures that, so far as known, were maintained under identical conditions.

Another of Robertson's experiments (Table II.) which he often quotes (Robertson, 1923, 1924*a, c*) as a proof of the allelocatalytic effect, may be compared with the data of Table I.

TABLE II.

Culture.	Initial Number.	24 hrs.	48 hrs.	Generations.	Average Gen. Time.
310*A*	1	2	16	4	12 hours
311*A*	2	3	120	6	8 hours

If the numbers of ciliates in cultures 310*A* and 311*A* are compared at the end of 48 hours, it is seen that there are 7.5 times as many cells in the two-animal culture. The average generation times are twelve hours for the one-animal culture and slightly more than eight hours for the two-animal culture. This is a difference of almost four hours, or a variation of ± 20 per cent. from the median of ten hours. Since Robertson's series of one animal cultures (Table I.), subjected to identical conditions, shows a variation of ± 31 per cent., it is impossible to consider this variation of ± 20 per cent. as being the result of any difference in division rate due to allelocatalysis. It is well within the normal variation and need not be accounted for otherwise.

Yocom (1928) isolated single individuals of *Oxytricha* into different amounts of sterile medium. He found a 14 per cent. higher division rate in the cultures of smaller volume and interpreted this as evidence of a catalyst of growth. In view of the previous discussion of bacterial food supply in this paper it is easy to see how such a small difference could arise. The food supply was less concentrated in the cultures of larger volume, and thus less readily available; hence the ciliates did not divide as rapidly as in the cultures of smaller volume.

Petersen (1929), in numerous isolation experiments with *Paramecium*, obtained results which are contradictory in that some experiments favor Robertson's view, while others are directly opposed to the theory of autocatalysis. In small volumes no acceleration was noticed in two-animal cultures, and washing produced no noticeable difference in division rate. However, when animals were isolated, washed or unwashed, singly, in two's, and in four's, into twenty drops of bacterized medium, the highest rate was observed in the four-animal cultures and the lowest in the one-animal cultures. Similar results were obtained with forty-drop cultures. Since Petersen's results argue both for and against allelocatalysis, an explanation of such contradiction must be found before her observations may be accepted as either supporting or opposing Robertson's theory.

The results of the writer's investigations offer no evidence for allelocatalysis. Except in one doubtful case, the cultures of higher concentration showed a growth rate equal to or less than that of the more dilute cultures. If an accelerating factor such as a "catalyst of growth" had been exuded during divisions, the higher concentration of this catalyst in the more concentrated cultures would have accelerated their growth, *especially after the first few divisions.* Since the growth rate was not accelerated during the first few days it must be concluded that no such " catalyst of growth " was present.

In most cases in which growth rate of the more concentrated cultures was less than that of the more dilute the difference became more and more pronounced as the culture grew older. This indicates an inhibiting factor in the more concentrated cultures, especially after the first few days. Part of the later differences are, of course, exaggerations due to the geometrical method of increase of early differences, but the causative agent of any difference, early or late, is unknown. The fact that most of the more concentrated cultures did not grow as rapidly as the more dilute is perhaps to be explained as due to a more rapidly diminishing food supply or a more rapid accumulation of waste products in the concentrated cultures or, more likely, to both these factors. This explanation is in accord with the results of Woodruff (1913), Cutler and Crump (1923*a*, *b*), Greenleaf (1926) and others on ciliates.

At the present time Robertson's theory of allelocatalysis appears to be upheld only by some of the experiments of Petersen (1929). The theory is opposed by the investigations of Woodruff (1911, 1913), Cutler and Crump (1923*a*, *b*, 1924, 1925), Greenleaf (1924, 1926), Calkins (1926), Myers (1927), some of the results of Petersen (1929), and by the investigations of the writer. In view of this bulk of evidence against the theory, and in view of the fact that the most striking results of Robertson are shown to be insignificant, the only conclusion to be drawn is that an allelocatalytic effect is not present in protozoan cultures.

Summary.

1. A method of obtaining accurate growth curves of euglenoid mass cultures is described and its efficiency demonstrated.

2. It is shown that under the conditions described no allelocatalytic effect is present in cultures of *Euglena* sp., and it is also shown that data of other workers which have been used to support the allelocatalytic theory do not support it but indicate the absence of any allelocatalytic effect.

LITERATURE CITED.

Calkins, Gary N.

'26 Biology of the Protozoa. Philadelphia and New York.

Cutler, D. W., and Crump, L. M.

'23*a* The Rate of Reproduction in Artificial Culture of *Colpidium colpoda*. Part I. Biochem. Jour., 17: 174–186.

'23*b* The Rate of Reproduction in Artificial Culture of *Colpidium colpoda*. Part II. Biochem. Jour., 17: 878–886.

'24 The Rate of Reproduction in Artificial Culture of *Colpidium colpoda*. Part III. Biochem. Jour., 18: 905–912.

'25 The Influence of Washing upon the Reproductive Rate of *Colpidium colpoda*. Biochem. Jour., 19: 450–453.

Fisher, R. A.

'25 Statistical Methods for Research Workers. Edinburgh.

Greenleaf, Wm. E.

'24 The Influence of Volume of Culture Medium and Cell Proximity on the Rate of Reproduction in Protozoa. Proc. Soc. Exp. Biol. and Med., 21: 405–406.

'26 The Influence of Volume of Culture Medium and Cell Proximity on the Rate of Reproduction in Infusoria. Jour. Exp. Zoöl., 46: 143–167.

Günther, Franz.

'28 Über den Bau und die Lebensweise der Euglenen, besonders der Arten *E. terricola, geniculata, proxima, sanguinea,* und *lucens* nov. spec. Arch. f. Protistenkunde, 60: 511–590.

Hargitt, G. T., and Fray, W. W.

'17 *Paramecium* in Pure Cultures of Bacteria. Jour. Exp. Zoöl., 22: 421–454.

Jahn, T. L.

'28 Relation of Volume of Culture Medium to Growth Rate of *Euglena*. (Abstract.) Anat. Rec., 41: 54.

Mainx, Felix.

'28 Beiträge zur Morphologie und Physiologie der Euglenen. I. Teil. Morphologische Beobachtungen, Methoden, und Erfolge der Reinkultur. II. Teil. Untersuchungen über die Ernährungs- und Reiz-physiologie. Arch. f. Protistenkunde, 60: 305–414.

Myers, E. C.

'27 Relation of Density of Population and Certain other Factors to Survival and Reproduction in Different Biotypes of *Paramecium caudatum*. Jour. Exp. Zoöl., 49: 1–43.

Parpart, A. K.

'28 The Bacteriological Sterilization of *Paramecium*. Biol. Bull., 55: 113–120.

Peters, R. A.

'21 The Substances Needed for the Growth of a Pure Culture of *Colpidium colpoda*. Jour. of Physiol., 55: 1–32.

Petersen, W. A.

'29 The Relation of Density of Population to Rate of Reproduction in *Paramecium caudatum*. Physiol. Zoöl., 2: 221–254.

Phillips, R. L.
'22 The Growth of *Paramecium* in Infusions of Known Bacterial Content. Jour. Exp. Zoöl., 36: 135–183.

Robertson, T. B.
'21*a* Experimental Studies on Cellular Multiplication. I. The Multiplication of Isolated Infusoria. Biochem. Jour., 15: 595–611.
'21*b* Experimental Studies on Cellular Multiplication. II. The Influence of Mutual Contiguity upon Reproductive Rate and the Part Played Therein by the X-substance in Bacterized Infusions which Stimulates the Multiplication of Infusoria. Biochem. Jour., 15: 612–619.
'22 Reproduction in Cell Communities. Jour. of Physiol., 56: 404–412.
'23 The Chemical Basis of Growth and Senescence. Philadelphia and London.
'24*a* Principles of Biochemistry. Philadelphia and New York.
'24*b* Allelocatalytic Effect in Cultures of *Colpidium* in Hay Infusion and in Synthetic Media. Biochem. Jour., 18: 1240–1247.
'24*c* The Influence of Washing upon the Multiplication of Isolated Infusoria and upon the Allelocatalytic Effect. Austral. Jour. of Exp. Biol. and Med. Sci., 1: 151–175.
'27 Some Conditions Affecting the Viability of Cultures of Infusoria and the Occurrence of Allelocatalysis Therein. Austral. Jour. of Exp. Biol. and Med. Sci., 4: 1–24.

Whipple, G. C.
'27 The Microscopy of Drinking Water. (Revised by G. M. Fair and M. C. Whipple.) New York.

Woodruff, L. L.
'11 The Effect of Excretion Products of *Paramecium* on its Rate of Reproduction. Jour. Exp. Zoöl., 10: 557–581.
'13 The Effect of Excretion Products of Infusoria on the same and on Different Species, with Special Reference to the Protozoan Sequence in Infusions. Jour. Exp. Zoöl., 14: 575–582.

Yocom, H. B.
'28 The Effect of the Quantity of Culture Medium on the Division Rate of *Oxytricha*. Biol. Bull., 54: 410–416.

HIBERNATION OF THE THIRTEEN-LINED GROUND SQUIRREL, *CITELLUS TRIDECEMLINEATUS* (MITCHILL). III. THE RISE IN RESPIRATION, HEART BEAT AND TEMPERATURE IN WAKING FROM HIBERNATION.[1]

GEORGE EDWIN JOHNSON.

Introduction.

The present study was taken up from 1915 to 1918 before the reports on waking of European rodents and bats (Horvath, '72, '78; Pembrey, '01, '03; Dubois, '96; Polimanti, '12; and others) had come to the attention of the author. While on this account many of the facts have been discovered independently by the author, the whole study has been reworked since investigation of the waking process was resumed in 1924, and it is here presented not only for its own value, but also in comparison with the work of others and as fundamental in the further study of hibernation and its possible causes. Brief reports on waking in a few individual ground squirrels have been given by Hahn ('14), Shaw ('25) and others, but the variability which has been found in the waking process has made an extended study necessary before any general description of it under different conditions of temperature and stimulation could be made.

In the first paper of this series (Johnson, '28) the conditions in hibernation and in normal activity were compared. The second paper (Johnson, '29) described two types of waking: (*a*) a relatively rapid awakening accompanied by trembling, following a *disturbing* of the animal by removing it from the nest and laying it on its side or back; (*b*) a more gradual awakening, usually without trembling, following removal *without disturbance* to a warm room, or following some handling after which it was placed back in the nest in the rolled-up position, and not removed from the cold room. In this type of awakening the raising of the head, the opening of the eyes and the moving forward often occurred about the same time, the state of hibernation apparently passing over gradually into normal sleep from which the animal awoke from some stimulus, external or internal. This is probably the type of waking found in nature, whereas the disturbed kind is that usually described in the literature.

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[1] Contribution No. 104 from the Department of Zoölogy, Kansas State Agricultural Experiment Station, Manhattan, Kansas.

This paper deals with the changes in respiration, heart beat and temperature in what must be considered the disturbed type of wakening, because the taking of the heart beat involved either the unrolling, or a more vigorous handling, of the animal.

Animals and Methods.

Some *Citellus tridecemlineatus tridecemlineatus* (Mitchill) and many *C. t. pallidus* (Allen) animals were used. The lower rate of heart beat which could not be obtained with a stetoscope, was secured by the use of a needle. At a rate of about 100 beats a minute the needle was removed and the stethoscope used. Some records were also taken by means of an electrocardiograph. It was hoped to obtain records of rise of heart beat in *undisturbed,* rolled-up animals with the electrocardiograph, but the placing of electrodes on the forelegs above the elbows apparently disturbed them even more than the needle, which could be inserted readily through the thoracic wall into the heart muscle after gently unrolling the animal and laying it on its back. For this reason the needle method appeared preferable and most of the heart beat records were taken with it. Temperatures were taken with small especially constructed thermometers but usually thermoelectrically, chiefly in the food pouch because this involved almost no stimulation. (See Johnson, '29, for further details of methods, and see discussion of Fig. 3, this paper, for further reasons for using pouch temperatures. As methods were modified in the progress of the work some discussion of them will be given within the paper.)

Miss Virginia Hanawalt, Messrs. Earl Herrick, E. Duane Sayles, Robert T. Hill and Mrs. Joanna Challans have assisted in various phases of the work. Dr. Ralph Major, of the University of Kansas Medical School, kindly extended the use of an electrocardiograph and he and his assistants aided in taking some heart beat records with this apparatus.

Changes in Respiration Rate in Waking from Hibernation.

From a rate of one half to four a minute in deep hibernation (Cf. Johnson, '29) the respiration increased distinctly in rate when the animal was disturbed and, as is seen in the graphs in Fig. 1, this increase usually continued till near the time of opening of the eyes, when very commonly the rate was about 150 a minute and usually ranged between 100 and 200 a minute. At about the time of opening of the eyes the respiration rate often fell distinctly if the animal was quiet. The very rapid shallow inspirations common in normal excited animals were not observed in awakening from hibernation. The graphs (Fig. 1, I.) show much variation in the rate of increase from minute to minute. This irregularity suggests the presence of a coarsely adjusted respiratory regulating mechanism, stimulation of which may produce over-aëration of the blood followed by decreased respiration for several seconds or even minutes. Such periods of decreased respiration following rapid respiration sometimes lasted as long as 30 or 40 minutes in animals awaking undisturbed in a warm room. No graphs are shown of these animals because heart beat and temperature records were not taken of them.

A definite correlation between rate of increase of respiration and higher room temperature is seen in the three groups of graphs in Fig. 1. The curves in I., *a*, taken in a room of about 2 to 12° C. rise slowly; those in I., *b*, taken in a room of 15 to 22° C., rise more rapidly; and those in I., *c*, from a room of 25 to 35° C., rise most rapidly.

Simultaneously with respiration there was taken the heart beat (Fig. 1, II., *a, b, c*) and temperature (Fig. 1, III., *a, b, c*). These animals were therefore disturbed slightly by a thermocouple in the food pouch and more by being laid on the back so that the needle might be inserted into the heart. They were also exposed more to the surrounding warm air in *b* and *c* (curves *E–L*) than if they could have been left rolled up. To avoid these sources of stimulation some records of respiration and temperature alone were taken, with the animal replaced in the nest rolled up in its natural position after the thermocouple was gently inserted into the food pouch. The head therefore was underneath and next to the cold wood shavings which constituted the nest. One of these

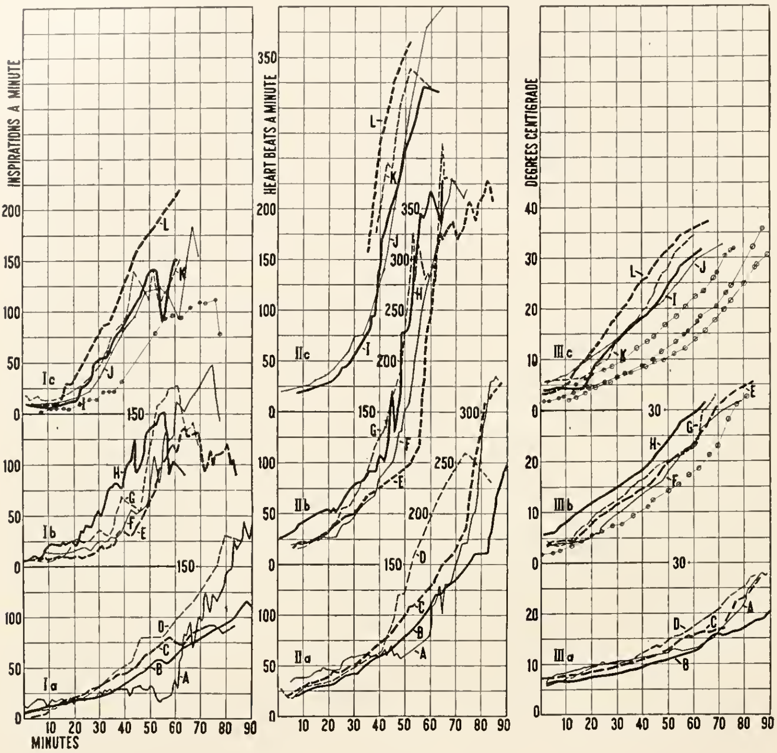

FIG. 1. Graphs of increase in respiration (I.), in heart beat (II.) and in temperature (III.), in a cold room of about 2° to 10° C. (*a*), in a room of about 15° to 22° C. (*b*), in a warm room of about 25° to 35° C (*c*). The curves belonging to one animal are designated by the same capital letter, thus I., *a A*, II., *a A* and III., *a A* represent the respiration, heart beat and temperature increases respectively in one waking in the cold room of the animal *A*. The fine lines connecting the rings in I., *c*, II., *b* and II., *c* are graphs of animals waking relatively undisturbed (thermocouple gently placed in the food pouch and the animal replaced rolled up in the nest). In the other graphs the animals were disturbed by being straightened out and by taking of heart beat in addition to temperature.

curves of relatively undisturbed awakening in a room of 23° C. was between *E* and *G* (Fig. 1, I., *b*). One in a room of 29° C. almost paralleled, but rose later than, *I* (Fig. 1, I., *c*). Another showed almost no rise for 50 minutes, then rose parallel to *I* in Fig. 1, I., *c*. A fourth, which rose about half way between the two mentioned, is shown with dots within circles in Fig. 1, I., *c*. The rolled-up position and the probable shortage of air together with the lack of continued stimulation, because of its normal position, probably accounted for the slow rise of the curve at first. Respiration curves of animals not disturbed but merely brought to a room of warm temperature lagged from about five to twelve minutes behind those shown, but paralleled them when the rise once started. In some such animals there were long periods of very slight respiration lasting from 10 to 20 or more minutes.

Changes in Heart Beat in Waking.

The heart beat of animals left in the cold room after inserting a needle through the thoracic wall into the heart sometimes failed to rise or even fell rarely in rate for a short time, but usually there was a gradual increase at first followed by a much greater increase in the latter part of the waking process as in Fig. 1, II., *a*. A few animals had a rapid rise within 20 minutes of the time they were disturbed. Such an early rise usually indicated that the animal was not very torpid.

When animals were transferred to a warm room of about 20° C. (Fig. 1, II., *b*) the rapid rise in heart beat and the peak of its rise both came sooner than in the cold room. The rise was still more rapid and the peak reached still earlier in animals taken to a room of about 29° C. (Fig. 1, II., *c*). Under all conditions of waking the heart beat was very rapid about or a little after the time of opening of the eyes and some records of 400 beats a minute were taken with a stethoscope. The rate fell shortly after this. The very rapid increase in rate just before opening of the eyes was not produced by holding the animal because it appeared in those animals which were not held before the eyes were opened. It did not appear to be produced by the pressure of the stethoscope against the thorax, because the same rise occurred in the records taken with the electrocardiograph (Fig. 5) where there was no stimulation of the animal except the pressure of the electrodes on the forelegs. Other evidence presented later in this paper also indicates that stimulation is effective only to a limited extent in

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speeding up the waking process and it appears very probably that there is always quite a marked rise in heart beat at this time in the disturbed type of awaking even if there is no stimulation at and just before the opening of the eyes.

In Fig. 1, II., *c*, *K* and *L*, two records taken with the stethoscope only are given, showing that the heart beat taken with a needle is similar in the latter part of the waking process at least to that in animals on which no needle was used.

THE RISE OF TEMPERATURE IN WAKING FROM HIBERNATION.

a. Comparisons of Œsophageal, Food Pouch, Deep Rectal and Anal Temperatures.—Graphs made of temperatures taken simultaneously in different regions of the body during waking from hibernation confirmed the observations of Dubois ('96) and others that the region near the heart warms up most rapidly. This is evident in Fig. 2, where it is seen that the deep œsophageal temperature (thermocouple inserted 5 cm. in from front of mouth, which would be to a point near the heart) rose slightly more rapidly than the temperature taken in the lower food pouch, as the animal lay on its side, which follows it very closely. The deep rectal temperature taken at a depth of 4 cm. and the anal temperature taken at a depth of 1.0 to 1.5 cm. showed almost no increase for some minutes and both lagged far behind the œsophageal and pouch temperatures until these two had almost reached their maximum some time after the animal opened its eyes and was able to move about. The anal temperature rose more slowly than the deep rectal. At other depths both in the œsophagus and in the rectum or colon slightly different curves than those shown would result. What is strikingly shown by this and five other graphs that agree with it is that the source of heat is in the thorax and as the heart and respiratory muscles are the ones which are contracting, it is evident that they are producing the heat. It is also clear that there is a marked distribution of this heat in the anterior part of the body since the pouch temperature follows that of the deep œsophagus closely but that there is a very slow diffusion of the heat to the abdomen, especially to the posterior abdomen, probably through a lack of circulation there. It is therefore a question where the temperature should be taken during the waking process.

Five other sets of graphs agree with those shown in Fig. 2 in that the pouch temperature is close to that of the œsophagus (maximum differences of 2°, 2°, 3°, 3° and 3°, respectively) in all cases following the œsophageal temperature more closely than did deep rectal or anal temperatures. In Fig. 3 the anal temperature is almost the same as the deep rectal in the cold room (see insert,

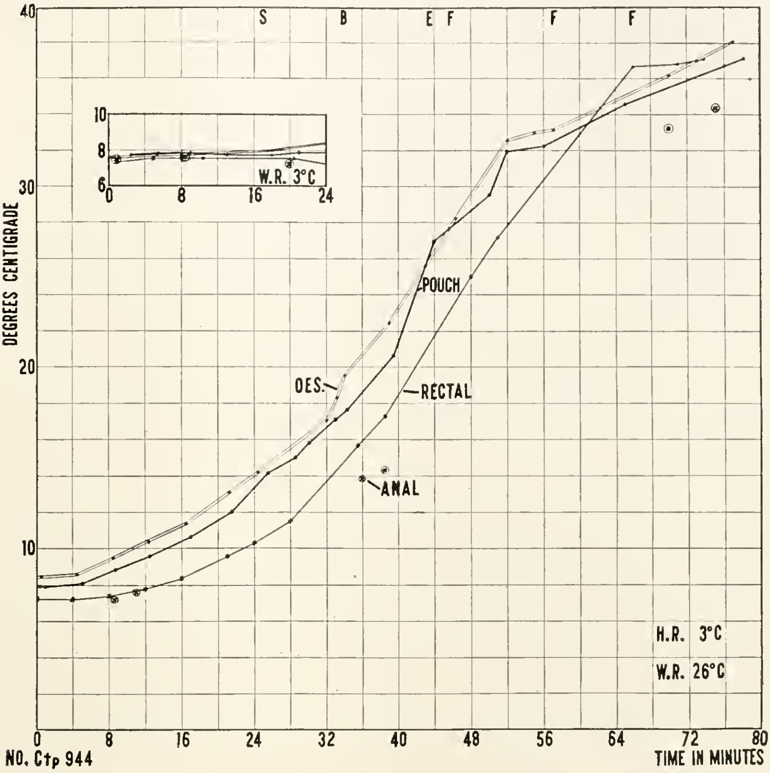

FIG. 2. Curves of the rise in temperature in the œsophagus (*œs.*) at a point 60 mm. in from the tip of the nose, in the food pouch (pouch), 43 mm. in the rectum (rectal), and 10–12 mm. in the anus (anal), all in one waking of *C. t. pallidus* 944. The insert gives observations in the cold room in which the animal was hibernating; and the main graph, the observations in the warm room. *H. R.*, hibernating room; *W. R.*, waking room; *S*, struggling; *B*, deep rapid breathing; *E*, eyes opened; *F*, fighting so that it had to be held or held down against the nest for the observation at that time. Note that the temperature of the pouch follows that of the œsophagus more closely than do the other two.

Fig. 2) and also for a time after removal to a warm room (main graph, Fig. 2). Only one of the other five graphs agrees with this. Four of them show that the anal temperature rose somewhat more rapidly at first (probably affected by the warmth of the room) and rather late in the waking period the deep rectal temperature rose above the anal. On account of this fluctuation and even more on account of the lag in the curves which prevents them from registering a change in heat production in waking, temperatures taken in the anus or rectum are not nearly so indicative of internal temperature as are either those of the pouch or the œsophagus.

In Fig. 2 the rise of the pouch and rectal above the œsophageal temperature was an irregularity not found in the other graphs and was probably produced by some unusual conditions, as the struggling of the animal at these times, or the use of a different thermocouple, one which was protected against biting, in the last part of this record.

It is evident that the deep œsophageal region near the heart is the center of heat formation and temperatures taken there would be very valuable if the animal were not disturbed in taking them. However, when records of relatively undisturbed awakening are desired œsophageal temperatures are out of the question. Pouch temperatures, while affected somewhat more by external temperatures than œsophageal temperatures, following œsophageal temperatures quite closely and doubtless are even more indicative of the internal temperature of the animal than is shown in the graphs which include also the œsophageal temperature, because pouch temperatures when taken alone can be secured with practically no handling and also because the thermocouple in the œsophagus stimulates the heart and respiratory muscles thereby causing the œsophageal temperature to rise abnormally fast, especially early in the waking period when waking is otherwise usually slow. It seems therefore that pouch temperatures taken with care give the rise of temperature in waking (produced by warming the animal or by disturbing it slightly at first) more accurately than does any other.

b. Rise of Temperature in Waking in a Cold Room of About 2° to 10° C.—Slight handling and the insertion of a thermometer bulb in the cheek pouch would produce waking in the cold room, and even very gently inserting a fine thermocouple into the cheek pouch would often but not always produce waking. In these studies the animal was held only when necessary and only late in waking. The process of waking in a cold room was studied in 12 graphs, four of which are given in Fig. 1, III., *a*. These four are selected to show the various types of curves rather than the most prevailing type. There was usually a slow rise in temperature for some time, then a rapid rise towards normal temperature, which was then approached more gradually, giving an S-curve somewhat similar to those illustrated by Dubois ('96) for the marmot. The temperature finally became fairly stationary if the animal was undisturbed, or continued to rise for some time if handled considerably (see Johnson, '28, for effect of handling). Variation in some of the waking curves was observed. In one the temperature rose almost uniformly during the whole period. In another there were two periods of rapid rise. In some graphs and in some records of not awakening where the animal was removed to a colder room the pouch temperature fell at first, and the internal temperature probably also did in these cases for the animals became more inert. When œsophageal temperatures were taken they rose steadily from the first because of the stimulation produced by the thermocouple, even when the pouch temperature was found to drop at first.

c. Rise of Temperature in Waking in a Room of 15° to 22° C.—Sixteen graphs were made of waking in a room of intermediate temperature, four of which are shown in Fig. 1, III., *b*. The effect of the warm surrounding temperature was evidenced by an increase in the rate of rise of the animal's temperature within a few minutes after the animal was removed from hibernating quarters to the warmer room. The rate of rise was not always rapid at first (Fig. 1, III., *F*), but in some cases it was slightly more rapid than near the time the animal had reached the temperature of the room (*H* and *E*), and quite frequently the curve approached a straight line.

To determine to what extent the rapid rise of temperature of the animal in a warm room was caused by the higher external temperature two dead animals were removed from the cold room to a warm room of about 25° C. One showed a marked rise in pouch temperature very similar to curves *E, G* and *H* (Fig. 1, III.) for the first 25 minutes. In the other the anal and pouch temperatures rose very close together and more rapidly at first than the deep œsophageal and deep rectal (4 cm.), but all rose more slowly than *E* and *G*. In all cases as the room temperature was approached the rise became slower.

From these and one more record in a warmer room, it is clear that the rapid rise in temperature of a torpid animal for the first few minutes after it is brought into a warm room is produced chiefly by the higher external temperature. As time passes the metabolism is greatly increased and becomes the chief cause and finally the only cause of further warming of the animal.

From the latest records taken it appears that curve *F* is characteristic of temperatures taken in a food pouch under the animal whereas curves *E, G* and *H* are more typical of those taken in the food pouch which was away from the nest. The four curves (*E–H*) were taken with the animals lying on the back with the food pouch away from the nest except probably *F*. Curve *F* is also quite similar to deep œsophageal temperatures as seen in seven graphs. An eighth graph of œsophageal temperatures formed a straight line.

d. Rise of Temperature in a Room of About 25° to 35° C.— Eighteen pouch temperature graphs of animals transferred from the refrigeration room to a warm room were made and studied. Some of the different types found are illustrated in Fig. 1, III., *I, J, K,* and *L*. A few of the curves (see *I*) had a period of rapid rise at first and one near the time of opening the eyes. Later work indicated that this is produced when the pouch is rather open causing warm air to enter the pouch. When a thin thermocouple was used and precautions taken not to force the mouth open most of the curves formed almost a straight line (*L*) or sometimes they rose slowly for a few minutes and then gradually more rapidly (*J, K*), especially shortly before opening the eyes, until the animal was awake, when the rate of rise slowed down and finally ceased.

e. Rise of Temperature in Relatively Undisturbed Awakening. —After gently inserting a thin flexible thermocouple into the food pouch of a torpid animal it was replaced in its nest in the rolled up position. This was done just before or just after removing the animal to a warm room. Heart beat was not taken in these animals. The temperature records are given in Fig. 1, III, *b* and *c* (dots connected by fine lines). With the head underneath against the cold nest and also with lack of stimulation from unusual position the rate of rise of temperature is quite slow at first resembling somewhat the curves of temperature of waking in a cold room.

CORRELATION OF RESPIRATION, HEART BEAT AND TEMPERATURE RISE IN WAKING FROM HIBERNATION.

In order to study the relations between the rise in respiration, heart beat and temperature, graphs of all three were combined from a single waking process initiated by slight handling or transference to a warm room. These combination graphs show correlations between the three curves, which rise more or less together. Waking in a cold room (0° to 8° C.) showed a very gradual rise in heart beat and respiration following insertion of a thermocouple for taking temperature and of a needle for counting heart beat (Fig. 3). The rise in temperature was even slower. In Ctp 128 the temperature fell for about the first seven minutes during which temperature was taken, for the animal had been transferred from a surrounding temperature of 6° C. to a waking room temperature of 0.2° C. (rising to 4° C. near the end of the awakening period) but the increasing heart beat and respiration maintained the temperature of the animal for the next ten minutes after which there was a slow but gradual rise similar to that in Ctp 740. In both of the graphs in Fig. 3 there are some irregularities in the temperature rise. In the curve for Ctp 740 the rapid rise at 68 and 71 minutes was evidently produced by vigorous fighting by the animal against the insertion of the thermocouple into its pouch. Ctp 128 was biting and fighting fiercely with the observer's hand just before it opened its eyes at 108 minutes, resulting in an unusual rise in pouch temperature followed by a drop such as was seen in no other graph. It is evident that when an animal is vigorously using its jaw muscles that an unusually high temperature might prevail in the cheek pouch for a few minutes, but the distribution of this heat over the body would soon cause a drop in the pouch temperature to near the average temperature of the anterior portion of the body. In the case of Ctp 740 the animal returned into hibernation and a second waking record was obtained under almost the same conditions of temperature. The graph was almost identical to that shown in figure 3.

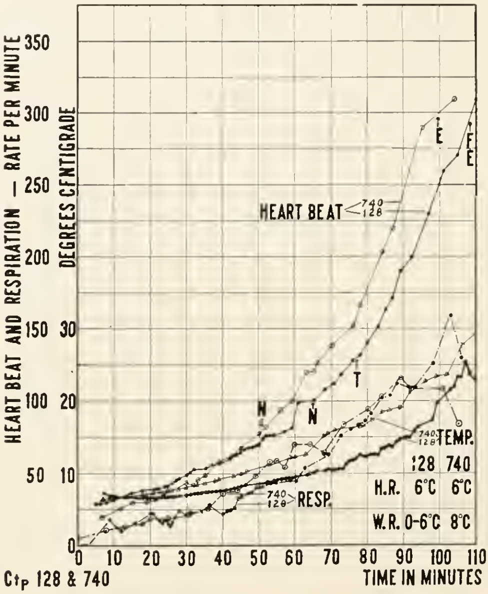

FIG. 3. Curves of rise in respiration, heart beat and temperature combined for study of correlations between them. Both *C. t. pallidus* 128 and 740 were removed from a refrigerator (H. R.) of 6° C. at 0 minutes and observations were immediately begun in rooms of 0° (rising to 6° by end of the record) and 8° C. respectively. *N*, needle removed and stethoscope used; *T*, trying to turn over; *E*, eyes opened; *F*, fighting. The dots on each graph indicate the actual readings taken.

Figure 4 gives the records of two animals waking simultaneously in a room of 30° C. under the same conditions except that no needle was used on animal Ctp 932. A general correspondence in rate of rise is seen for the three processes in each animal and also between those of the two animals. Ctp 932 was distributed five minutes later than Ctp 947 but it had a higher initial temperature so that its waking process was slightly the more rapid of the two. If the curves of Ctp 932 were moved to the right until the initial temperatures of the two animals correspond then the temperature and heart beat of the two would be almost identical. The respiration curve of Ctp 932 would lag behind at first but be higher at the end than that of Ctp 947.

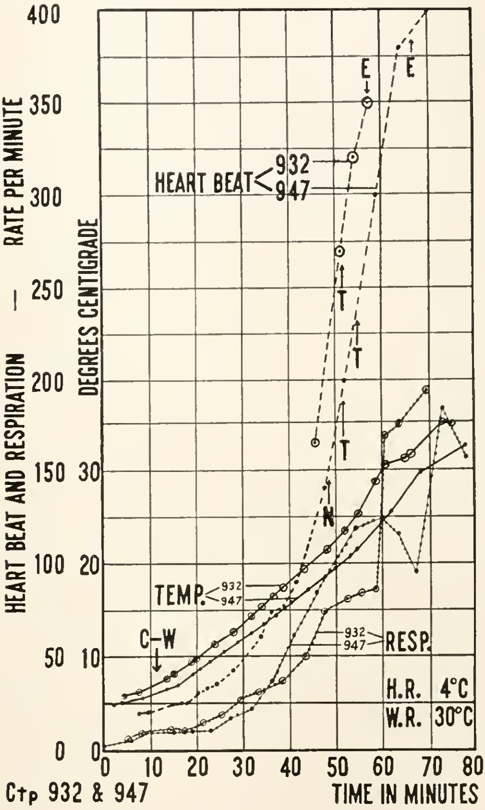

FIG. 4. Curves of rise in respiration, heart beat and temperature of *C. t. pallidus* 932 and 947 taken at the same time. The heart beat of 947 was taken with a needle to *N*, then with a stethoscope. A stethoscope alone was used on 932. *H. R.*, hibernating room; *C. W.*, point of transfer of animals from a room of 10° C. to a warm room (*W. R.*) of 30° C.; *T*, trying to turn over on feet; *E*, eyes opened.

Figure 5 illustrates two cases of waking of *C. t. pallidus* animals in which the heart beat was determined with an electrocardiograph. The electrodes were wound about the fore legs of Ctp 734 three minutes before the beginning of the record as given in the graph. The animal was transferred from a temperature of

7° to one of 17° C. at three minutes in Fig. 5. When the electrodes were placed on the fore legs the animal moved only very slightly but the respiration rose from an average of about two to seventeen a minute. Owing to the fact that all the observations were made by the author unaided and also to the fact that the

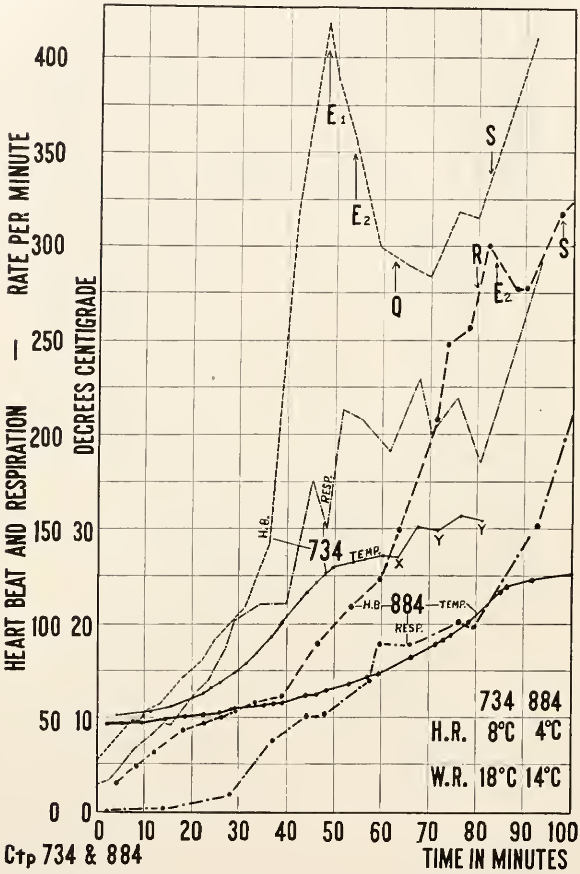

FIG. 5. Curves of rise in respiration, heart beat and temperature of *C. t. pallidus* 734 and 884, in which the heart beat was determined with the aid of an electrocardiograph. Both animals were straightened out, electrodes placed on the fore legs, thermocouple inserted into the food pouch and the animal laid on its back or side in the nest. *H. R.*, hibernation room; *W. R.*, waking room; E_1, first eye open; E_2, both eyes open; *Q*, quiet; *S*, struggling; *R*, righting, turning over on feet; *x*, thermocouple partly out of food pouch; *y*, thermocouple probably partly out.

thermocouple did not remain completely in the pouch after the animal opened its eyes the temperature curve is not smooth as in practically all other graphs. It should be noted that this record is carried further past the opening of the eyes than in the other graphs. This accounts for the fluctuations in heart beat and respiration in the last 50 minutes.

Ctp 884 was removed from an outdoor temperature of 4° to a room temperature of 13° C. at the beginning of the record shown in Fig. 5. This animal was not disturbed much by the electrodes being placed on the fore legs. Probably partly on this account and partly on account of the colder room it woke more slowly than Ctp 734. However, internal conditions may have been influential also, as indicated in some other waking records in which an animal would wake much less readily than usual.

It is to be noted in all cases (Figs. 1, 3, 4) that the heart beat rises very rapidly once it has reached 200 or 250 a minute shortly before the opening of the eyes.

Since some observers (*e.g.*, Hall, '32) have intimated that a rapid increase in respiration as soon as the animal is disturbed is the cause of waking, an attempt was made to determine whether heart beat or respiration took the lead in waking. Fourteen graphs of waking in a cold room (1° to 12° C.) show the heart beat and respiration rising before the temperature in practically all cases. It was difficult to determine accurately whether heart beat or respiration rose first, but in about eight or nine cases the heart beat appeared to rise first, and in two or three cases respiration appeared to rise before the heart beat.

In the warm room of about 29° C. the temperature rose first in practically all of the eight graphs studied, and heart beat rose before respiration in all but one case. In a room of about 20° C. the temperature rose first in four out of eleven cases, temperature and heart beat rose together in three others and in one or two cases the heart beat rose first. Respiration was usually last to rise (seven in eleven cases). In two or three cases all three rose at about the same time.

In an electrocardiograph record of a *C. t. pallidus* the heart beat appeared to rise before the respiration when disturbed outdoors (5° C.) and fell first of the two as the animal went back into deep hibernation. Four hours later when removed to a room of 18° C. heart beat again rose in advance of respiration. In this animal the heart missed a beat more or less frequently, as seen in Fig. 6 which is shown here because the missing of beats was not uncommon and sometimes caused abnormal awakening. (No such records have been included elsewhere in this paper.) After reaching a rate of 63 a minute at 7:25 P.M. the rate fell to 50, 44 and 20 a minute at 7:29, 7:35 and 7:42 P. M., respectively (see Fig. 6, *a*, *b*, *c*).

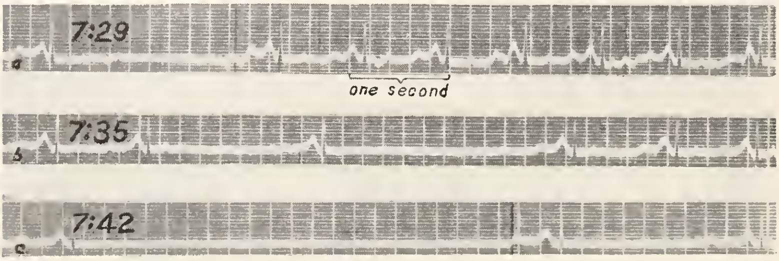

FIG. 6. Electrocardiograms of Ctp 938 showing how heart beat may fall abnormally in rate from a missing of beats after waking from hibernation has begun.

The respiration increased some after the heart beat had begun to fall, but 18 minutes after the highest heart beat the respiration had reached its highest point and took a rapid fall, remaining low in spite of the gradual warming of the animal by the room temperature (18° C.).

On a previous day (on which it showed no irregularity of heart beat) the same animal after partially awakening in a warm room showed an earlier drop in heart beat rate than in respiration when the room was rapidly cooled. During two hours of gradual warming of the room from 12° to 22° C. the heart beat rose gradually from 12 to 36 while the respiration continued at about 5 a minute. Near the end of that time the respiration showed one rise to 20 and another to 15 a minute.

While it is difficult to attribute a leading rôle to either respiration or heart beat in waking, it seems that the heart usually initiated the process to a greater extent than respiration when the animal was disturbed in a cold room. In a warm room waking was chiefly induced by a rise in body temperature followed by an increase first in heart beat and then in respiration.

In several records respiration rose when the animal was disturbed but fell again in a few minutes, whereas the heart once started to beat faster tends more to accelerate and produce waking, as if once started to beat faster it could not decrease in rate again till waking has occurred.

The Effect of Stimulation upon the Rate of Waking.

Since it was evident that waking usually followed disturbing the animal by taking its temperature even in the cold room, it was thought that stimulation of the torpid animal would show whether heart beat or respiration was first to respond and therefore leading in the waking process. The results of a number of experiments were not very clear cut. It became evident that while stimulation of some sort usually starts the waking process, the rate of waking is not increased in proportion to the increase in amount of stimulation.

Electrical stimulation (rapid make and break shocks with a Harvard inductorium using one dry cell of 1.5 volts with the secondary coil of the inductorium set at 6 cm.) was applied to four animals a total of eleven times for a few seconds each time. Heart beat increased in four cases, decreased in three, and remained the same in three. The respiration rate increased in five cases, decreased in four cases and did not change in two cases. In one animal which did not awaken the respiration fell to 0 for a minute following six out of seven stimulations. Heart beat also was retarded in three out of the seven stimulations.

Stimulation by dropping a 5 gram weight on the animal every ten seconds from a height of five inches appeared to slightly accelerate the rate of waking, but this was only slight for the opening of the eyes occurred about the same time as in the controls in three experiments of four to eight animals each.

The continuous ringing of an electric door bell suspended near the animals had no noticeable effect on three animals as compared with their controls. Ringing for six-minute periods inhibited respiration and heart beat for a minute after the bell was started for the first two ringings. In another animal which was waking

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more rapidly than usual the sound of the bell did not check heart beat and apparently accelerated respiration.

Handling the animal did not always produce waking. In a room where the temperature was falling the insertion of a thin flexible thermocouple into the cheek pouch of a torpid animal did not usually cause it to wake, though a slight temporary rise in respiration and temperature was often observed. A thermometer bulb inserted into the cheek pouch produced waking. Some animals appeared to wake more readily than others, though this could often be ascribed to a difference in body temperature at the start. One castrated male ground squirrel began to awaken in the cold room, but soon respiration, heart beat and temperature all fell and it became completely torpid again. The animal was disturbed only at first.

The Effect of a Near Freezing Temperature on Waking.

Since Horvath ('81), Dubois ('96), and Pflüger and others (according to Merzbacher, '04) found that temperatures near 0° C. caused hibernating mammals to awake and since ground squirrels appeared to me to hibernate very well below 5° C., two experiments were performed to determine whether there was a difference between species in this regard. At one time six *C. t. pallidus* were transferred from a warmer part of the refrigerator to a colder part (4° at first, falling to 2° C. in 4½ hours). All were still torpid in 85 minutes, but Nos. 1 and 2, which had a higher respiration rate (20 a minute) than the others at first, were awake, and Nos. 3 and 4, which had been deeply torpid, were waking, at 4½ hours. Left over night Nos. 1 and 2 were partly torpid again at a refrigerator temperature of 1° C., and Nos. 3 and 4 were now very deeply torpid, as was also No. 5, but No. 6 was partly torpid and woke before the next day. On the third day Nos. 1, 4 and 5 were very deeply torpid at a refrigerator temperature of 0.85° C. and No. 3 was partly torpid. On the fourth day Nos. 1, 3 and 4 were torpid at a refrigerator temperature of 0.3° C.

At another time the cages of three *C. t. pallidus* were placed on the floor of the very well insulated room at an air temperature of 1° C. and three others left in the refrigerator at 2° to 3° C. All were deeply torpid at first except that one of those removed to the floor was breathing deeper than the others. This and one other on the floor and one in the refrigerator woke within eight hours. The third one on the floor was still torpid the next morning at a room temperature of 0° C., but began to awake in the afternoon. Two in the refrigerator did not wake the first two days. Transferred to the floor one woke the following day.

While many of the animals in these experiments woke from hibernation when transferred to a room of within a degree of freezing, this did not occur to the extent claimed for the marmot by Dubois ('96), nor did this temperature hinder a return to the hibernating state in ground squirrels. An examination of his plates 4 and 5 show that the buccal temperature rose from the first. This fact suggests strongly that waking was produced partly by the mechanical stimulation involved in taking the rectal temperature and in transferring the animal to the colder room. The same two sources of stimulation were present in Horvath's ('81) experiments. Further evidence that waking from hibernation does not always occur was found in the death of a number of hibernating ground squirrels in the early part of the work when attendants permitted the room temperature to fall to or below freezing.

Discussion.

A continuance of hibernation appears to be dependent upon the absence of external and internal stimuli, for waking is readily induced by mechanical or electrical stimuli or by warming. Since some animals wake more readily than others internal conditions appear also to influence waking. This is also indicated by the awakening at intervals of a few days of many hibernating animals.

Heart beat appears to increase somewhat more than respiration at the beginning of waking and, therefore, seems to have more influence on waking than does respiration. The interesting condition of inhibition of respiration producing inhibition of heart beat in the duck shown by Dooley and Koppanyi ('27) was kept in mind during this study of waking from hibernation, but heart beat seemed to rise independently of respiration, not only in ordinary waking but also in a case where the thorax was cut open so that the animal could not secure air. This animal had a higher rate of heart beat when it was warmed, rising from 4 to 28 a minute in 37 minutes in one animal transferred from a surrounding temperature of 4° to one of 18° C. Such an animal could probably not become much warmer than the room because of the lack of oxygen in its blood.

At no time in waking were heart beat and respiration at the same rate, but sometimes the two would occur together, a condition claimed to be common in deep torpor by Dubois ('96) in the marmot. He also thought that the heart beat was augmented when it occurred at the same time as an inspiration suggesting a causative relation of respiration to heart beat. No such increased beat of the heart was apparent when it occurred at the same time as a respiration in the torpid ground squirrel.

The rise of temperature in a torpid animal in a cold room is slow at first because respiration and heart beat increase only very gradually and, therefore, there is little increase in heat produced. It is generally considered (Bayliss, '18, p. 455) that muscular contraction is the "chief if not the only, source of heat of practical importance to the animal organism."

Since contact or electrical stimulation usually causes waking of the torpid animal, the question arises why persistence of stimulation during waking does not speed up the process greatly. That it does not do this at all in proportion to the amount of stimulation, suggests the "all or nothing" principle. This principle would not seem to hold in an exact way, but waking is nearly always completed when once started, and the rate may not be increased much by added stimuli. One reason that the rate is not modified greatly by stimulation is probably the inability of muscle to contract rapidly when cold as observed by electrical stimulation of the muscles through the skin. The acceleration of the waking process by warming and the retardation by cooling the animal support this, as does also the regaining of motility in the anterior (warmer) regions of the body at an earlier time than in the posterior (colder) portion of the body in waking. At the very beginning of waking from deep hibernation there is usually no response to even severe mechanical stimuli like cutting through the body wall, indicating that such stimuli do not pass through the central nervous system, but as waking begins and while the animal is still cold (10–15° C.) coördinated responses to electrical and mechanical stimuli appear. These responses are sluggish but fairly vigorous, and lead an observer to expect the stimulated animals to wake up much more rapidly than the controls.

Another factor in the production of a rapid rise of metabolism may be the temperature control mechanism. This appears to be only slightly active in the first part of waking and in quiet awaking does not appear to be so active as in the latter part of stimulated awakening where the shivering and trembling probably produced by the heat regulating system doubtless aid in the production of the very rapid rise in metabolism shown in most of the graphs in this paper. That the heat regulating mechanism is not so active in animals waking undisturbed in a warm or cold room may be indicated by the almost entire absence of shivering and shaking.

The production of a torpid state in cats by means of anæsthesia by Britton ('22) and the gradual recovery that resembles waking from hibernation tends to bear out the idea that the muscles of the heart and thorax are inhibited by cold and have grades of functioning according to their temperature, which also appears to be true of cold blooded animals. Britton could not produce a state of artificial hibernation but found that the cats would either die or very slowly awake from a body temperature of about 19° C. in a room of the same temperature. An experiment in which the temperature of the ground squirrel was lowered by means of ether anæsthesia to 22.8° C. was performed by the author before he knew of Britton's work. Spontaneous awakening took place. Many interesting comparisons might be worked out between the cooling of a cat or other homoiothermal animal and of a hibernating mammal. It should be noted that Horvath ('81) found that the hibernating animal had the ability to survive when the body temperature is lowered in ice water to much below 19° C. without artificial respiration, whereas non-hibernating forms (dog, rabbit) died if subjected to temperatures below 19° C. without artificial respiration (Horvath, '76). If artificial respiration was kept up the rabbit heart stopped beating at 9° C., whereas in the hibernating animal it did not stop at 4° or even 2° C. (Horvath, '81).

SUMMARY.

The increase in respiration, heart beat and temperature as shown in graphs is very gradual at first in waking in a cold room of 0° to 8° C., whereas in a warm room of near 30° C. the process is more rapid from the first. In both cases there is a very rapid rise of all near or at the time of the opening of the eyes when the respiration rate commonly ranged between 100 and 200 a minute, and the heart beat rate usually ranged between 300 and 400 a minute, and the temperature between 20° and 34° C. The anterior part of the animal wakes more rapidly than the posterior regions, and œsophageal and food pouch temperatures indicate the increase in metabolism more accurately than deep rectal or anal temperatures. There is a general correlation between the curves of rise of respiration, heart beat and temperature. In the production of waking heart beat appears to be somewhat more effective than respiration. Waking may be induced by stimulation but its rate is not proportional to the duration or intensity of the stimulation applied, except in the case of a surrounding high temperature. Transference to a room of about 0° C. appears to stimulate waking in some but not in all torpid ground squirrels. This temperature does not prevent hibernation.

LITERATURE CITED.

Bayliss, W. M.
'18 Principles of General Physiology. Longmans, Green & Co., London.
Britton, S. W.
'22 Effects of Lowering the Temperature of Homoiothermic Animals. Quart. Jour. Exp. Physiol., 13: 55–68.
Dooley, Marion S., and Koppanyi, Theodore.
'27 The Cause of Bradycardia Accompanying Postural Apnea in the Duck. Anat. Rec., 37: 123–124. Abstract.
Dubois, Raphael.
'96 Physiologie compareé de la marmotte. Annales de l'Université de Lyon. Paris.
Hall, Marshall.
'32 On Hibernation. Phil. Trans., Pt. 1, 335–360.
Hahn, W. L.
'14 Hibernation of Certain Animals. Pop. Sci. Mo., 84: 147–157.
Horvath, Alexis.
'72 Zur Physiologie der thierischen Wärme. Centralbl. f. med. Wissencsh., Nr. 45–47, 706–708, 721–724, 737–739.

'76 Zur Abkühlung der Warmblüter. Pflügers Arch. f. Physiol., 12: 278–282.

'78 Beitrag zur Lehre über den Winterschlaf. Verh. d. Physik.-med. Ges., N. F. 12: 139–198 and 13: 60–124.

'81 Einfluss verschiedener Temperaturen auf die Winterschläfer. Verh. d. Physik.-med. Ges. in Wurzburg, N. F. 15: 187–219.

Johnson, George E.

'28 Hibernation of the Thirteen-lined Ground Squirrel, *Citellus tridecemlineatus* (Mitchell); I. A Comparison of the Normal and Hibernating States. Jour. Exp. Zoöl., 50: 15–30.

'29 Hibernation of the Thirteen-lined Ground Squirrel, *Citellus tridecemlineatus* (Mitchell); II. The General Process of Waking from Hibernation. Amer. Nat., 63: 171–180.

Merzbacher, L.

'04 Allgemeine Physiologie des Winterschlafes. Ergebn. d. Physiol., 3 (2): 214.

Pembrey, M. S.

'01 Observations upon the Respiration and Temperature of the Marmot. Jour. Physiol., 27: 66–84.

'03 Further Observations upon the Respiratory Exchange and Temperature of Hibernating Mammals. Jour. Physiol., 29: 195–212.

Polimanti, O.

'12 Il letargo. Roma.

Shaw, William T.

25 Observations on the Hibernation of Ground Squirrels. Jour. Agr. Res., 31: 761–769.

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BIOLOGICAL BULLETIN

THE SO-CALLED CENTRAL BODIES IN FERTILIZED *ECHINARACHNIUS* EGGS.

II. The Relationship between Central Bodies and Astral Structure as Modified by Various Fixatives.[1]

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Statement of the Problem.

It is generally agreed among cytologists that central bodies (centrosomes, centrioles) are the formative foci about which arises the mitotic mechanism of the animal cell. There is diversity of opinion, however, concerning whether or not they exhibit genetic continuity as individualized structures from cell to cell and from generation to generation. The behavior of central bodies in echinoderm fertilization has been regarded, heretofore, not only as supporting the theory that they are the formative foci of asters, but also as yielding evidence in favor of their genetic continuity from generation to generation.[2]

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[1] The material was secured at the Marine Biological Laboratory, Woods Hole, Massachusetts.

[2] A review of the literature concerning central bodies in echinoderm fertilization, together with a brief discussion of terminology, will be found in the first paper of this group (Fry, '29, pp. 101–109). A further discussion will be found in the present study, pp. 143–147.

There is much confusion concerning terminology in the literature because a given term is used by various authors with different meanings. In the present group of papers the term *centriole* applies only to a minute period-like type of central body; the term *centrosome* applies only to a larger, more diffuse type that is often vaguely delimited; the term *central body* is a broad one including both the others. It is unfortunate that in the previous paper (Fry, '29) the inclusive non-committal term *central body* was used most frequently. It is better to avoid this ambiguous term as much as possible, using instead the more specific words *centriole* and *centrosome.* This procedure is carefully followed in the present paper.

In the preceding investigation (Fry, '29) central bodies in *Echinarachnius* eggs were studied at various intervals from fertilization to first cleavage, after fixation with Bouin's fluid. The following conclusion was reached, based upon a detailed analysis of over 900 eggs. *The so-called centrosomes of fertilized Echinarachnius eggs are nothing but coagulation artifacts of ray substance at the astral center, produced by the fixative only during those periods when rays are well formed.* Both sperm-asters and cleavage-amphiasters pass through an early vague-rayed period during which there are no centrosomes; not until the rays become clear do centrosomes appear; when such well formed rays fade the centrosomes disappear. These facts are in harmony with central body phenomena during the history of cytasters in artificially activated eggs (Fry, '28, Chart III.). *The so-called centrioles of fertilized Echinarachnius eggs are nothing but cytoplasmic granules.* They are usually abundant among the astral rays but are generally absent in the mid-region. They occasionally occur there, however, in various positions and in various numbers. In a very limited percentage of cases such granule configurations simulate centrioles.

If the hypothesis is correct that centrosomes of fertilized *Echinarachnius* eggs are actually coagulation artifacts of the focal point of clearly fixed rays, then no matter by what means astral structure is modified, centrosomes should be present in coagulated asters only when rays are clearly fixed, and they should be absent when rays are fixed vaguely or not at all. This relationship should hold good whether ray structure is modified by successive phases in the normal astral cycle, or by different coagulation effects of various fixatives, or by modifications due to environmental factors, or by other causes.

In the previous study of the effect of different mitotic stages upon rays and central bodies the environment is constant, the living eggs being kept in normal sea water at 20° C. The fixative is also constant; Bouin's fluid is used because it is one of the standard reagents that clearly coagulates rays. The variable is the astral structure: different kinds of ray configurations, varying from vague to clear, occur during the successive stages of the astral history, thus giving an opportunity to study the relationship between the presence of centrosomes and the occurrence of clear rays reaching the center.

In the present study the environment is constant as above. The astral stage is also constant, only metaphase asters being selected for study because when properly fixed they show clear rays and typical echinoderm central bodies. The fixative is the variable: different kinds of ray configurations, varying from vague to clear, are produced by a wide variety of coagulating agents, thus giving another opportunity to study the relationship between clear rays and centrosomes.

In this experiment the fertilized eggs were kept in normal sea water at 20° C. They were simultaneously placed in a wide variety of coagulating agents one hour after insemination, the time when metaphase asters are most abundant. A study of such asters with water-immersion objectives shows that they have a similar structure in the living condition: the outer portion has a clearly radiate configuration, although the details are indistinct; the center is hyaline and apparently structureless. Hence the modifications of metaphase astral structure analyzed in the present investigation are due to the differing coagulation effects of various fixatives acting upon metaphase asters that were relatively similar in the living eggs.

If the proposed hypothesis is correct that centrosomes in fertilized *Echinarachnius* eggs are nothing but coagulation artifacts of the focal point of clearly fixed rays and have no existence as individualized bodies in the living condition, then in the present study they should be present only in the case of those reagents that coagulate the radial configuration of the living metaphase asters in such a manner that the fixed asters have clear rays. They should be absent when rays are fixed vaguely or not at all. Furthermore, if centrosomes are actually coagulation products of the inner ends of rays, they should also show a very great diversity in structure in the various reagents. Such modifications should be far more diverse than those that would be produced by different fixatives acting upon a component such as chromosomes which have an actual existence as formed bodies in the living cell.

The Method of Study.

Fertilized *Echinarachnius* eggs were simultaneously fixed in 128 reagents one hour after insemination. These fixatives comprise the standard ones, together with their components used separately, as well as various unusual combinations of reagents.[3] In the case of each fixative, about ten metaphase figures, chosen at random, were each measured and analyzed with reference to the twenty-two points listed in Table I. Upon the basis of this preliminary examination, the coagulation products of twenty-seven fixatives were chosen for illustration, and in the case of each one, an additional fifteen metaphase figures were similarly analyzed.[4] Those selected for intensive study were chosen to illustrate the following points: (1) to show the effects of representative standard fixatives, *i.e.,* sublimate-acetic (Fig. 3), Carnoy (Fig. 11), Petrunkewitsch (Fig. 12), Burckhardt (Fig. 15), Zenker (Fig. 16), Flemming (Fig. 19), and Bouin (Fig. 27); (2) to show the effects of special fixatives devised to demonstrate Golgi-bodies and chondriosomes, *i.e.,* Kopsch (Fig. 17), Benda (Fig. 18), Flemming (Fig. 19), Hermann (Fig. 20), formol, neutralized (Fig. 21), and Regaud (Fig. 22); (3) to show the ef-

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[3] A joint study with Dr. H. H. Darby is now in progress concerning the effect of pH upon the coagulation of asters in many fixatives. Some of the slides of that study were used in the present investigation; in such cases the fixative is at its normal pH.

[4] Appreciation is expressed to Miss Sara Jane Reynolds for her help as research assistant in making the thousands of measurements and observations involved in the preparation of this paper, as well as the others of the series.

Aster.

Shape and dimensions.
Whether or not differentiated into zones: in region about central body; in outer part.
Stain in contrast to that of the cytoplasm.

Astral Rays.

Whether clearly or vaguely fixed.
Whether delicate or coarse.
Whether a continuous line or a series of granules.
Whether relatively straight and largely separate from each other, or crooked and anastomosing.

"Centrosome."

(*i.e.*, large diffuse portion of central body.)

Shape and dimensions.
Stain in contrast to that of ray area.
Physical structure, *i.e.*, whether entirely homogeneous, or coarsely granular, or mulberry-like, or reticular, or vacular, etc.
Whether or not traversed by rays.
Whether or not clearly delimited from the ray area.

"Centriole."

(*i.e.*, small, period-like granule, or granules).

Number and size.
Location within the "centrosome."

Cytoplasmic Granules.

Abundance among rays: in outer part of aster; in inner part.
Comparison with "centrioles."

Spindle.

Length and width.
Stain in contrast to that of aster.
Structure of spindle fibers in contrast to that of astral rays.

Chromosomes.

Size.
Exact location and grouping.

Table I.

Points with reference to which cleavage (metaphase) figures are measured and analyzed in the case of each fixative.

fects of the various components of the above fixatives when used separately, *i.e.*, corrosive sublimate (Fig. 1), chloroform (Fig. 7), absolute alcohol (Fig. 9), potassium dichromate (Fig. 13), chromic acid (Fig. 14), osmic acid (Fig. 17), formol, neutralized (Fig. 21), formol, acid (Fig. 24), acetic acid (Fig. 25), and picric acid (Fig. 26); (4) and finally, to show all the types of coagulation products present in the 128 fixatives originally examined.

In the previous study in which Bouin's fluid was used the metaphase asters fall into two clearly demarked classes. In "early" metaphase asters (Fry, '29, Fig. 21) the chromosomes of the metaphase plate are in a tangled group; the rays are very delicate; there is no centrosome. In "late" metaphase asters (Fry, '29, Fig. 22) the chromosomes of the metaphase plate are aligned in two closely approximated groups that are ready to separate; the rays are coarse; there is a pleuricorpuscular centrosome.[5] In the case of many fixatives of the present study there are no differences between "early" and "late" metaphase stages, but whenever such differences do exist, the "late" metaphase asters were studied.

In many of the fixatives selected for illustration all the metaphase ("late") asters fall into one class with reference to the central body (centrosome). In the remainder there are two or three classes; in such cases that class occurring in largest numbers is illustrated. If more than one type of coagulated metaphase aster occurs in a fixative, this situation is usually caused by various degrees of destaining among different eggs of the same slide, a factor that may produce marked differences in appearance. More rarely the differences are due to the fact that the metaphase asters of different eggs are not coagulated in exactly the same manner; this is apt to occur in those fixatives that coagulate rays very faintly.

The formulae used are those listed in Lee's "The Microtomist's Vade Mecum." Eggs were left in the fixative about two hours. The methods of running up are those of the usual practise as described by Lee. In the case of the Golgi-body fixatives (Kopsch,

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[5] The previous illustration of metaphase asters after Bouin's fixation (Fry, '29, Fig. 22) shows the centrosome too dark and too sharply demarked. Such asters are correctly illustrated in the present paper (Fig. 27).

Fig. 17; Benda, Fig. 18; formol, neutralized, Fig. 21; and Regaud, Fig. 22), after fixation the eggs were osmicated at various intervals from one to fourteen days at room temperature; they were immediately run up and embedded without being allowed to stand in the alcohols. Eggs fixed in those reagents used for the demonstration of chondriosomes (Benda, Fig. 18; Flemming, Fig. 19; Hermann, Fig. 20; and Regaud, Fig. 22) were chromated at various intervals from three to ten days. Eggs were sectioned 5 μ thick. They were stained with Heidenhain's haematoxylin. They were studied at 750 × magnifications.

The figures (pp. 140–141) are arranged in horizontal columns with reference to various fixative groups, such as those using mercuric chloride, alone and in combination (Group I.); those using chloroform, alone and in combination (Group II.); etc. Underneath each figure is given the formula, and the name, if such occurs, by which the fixative is generally known. In addition to the horizontal arrangement with reference to fixatives, there is a vertical grouping with respect to whether or not rays are clearly fixed. The fixatives included in the narrow left hand column fix rays very vaguely or not at all; those in the broad right hand column fix rays clearly, whether they be coarse or delicate.

The figures are 650 × enlarged. The diameter of the coagulated aster in Fig. 3, for example, is 28 μ. In each figure, every dimension and the physical appearance of each part is an average of all the observations made with reference to the points listed in Table I., in all the individuals studied, belonging to that class of ("late") metaphase asters occurring in largest numbers, in that fixative. The chromosomes, however, are an exception to this, as in each case they were drawn from a single cell and were studied only superficially.

The method of study used in the present work is the same as that previously described (Fry, '28, pp. 387–392; '29, pp. 109–113). At each stage, using a given fixative, there is selected at random a sufficiently large number of cells to constitute an adequate random sample. They are all measured and analyzed in the greatest detail possible, and are classified in their various groups; the percentage of each class is noted. The interrelationships of the varying factors are observed in each group. This method is repeated in a number of diverse fixatives. Any conclusion is based upon a consideration of many classes of coagulation products, knowing the percentage of each, and the manner in which the different variable factors are interrelated in the various classes.

THE EFFECTS OF VARIOUS FIXATIVES UPON THE RELATIONSHIP BETWEEN CLEARLY FIXED RAYS AND CENTRAL BODIES IN METAPHASE ASTERS OF FERTILIZED *ECHINARACHNIUS* EGGS.

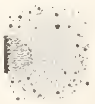
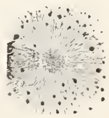
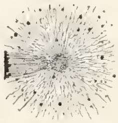
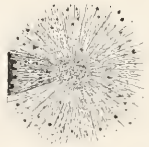
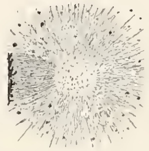
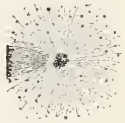
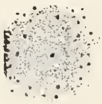
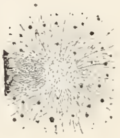
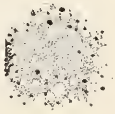
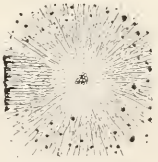
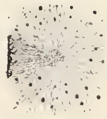
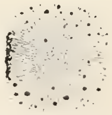
When metaphase asters of the first cleavage figure in *Echinarachnius* eggs are coagulated by a variety of fixatives, centrosomes are present only in the case of those reagents that fix rays clearly whether they be coarse or delicate; they are absent when rays are fixed vaguely or not at all (Figs. 1–27). This result is in harmony with that obtained in the study of the effect of fixatives upon cytasters in artificially activated eggs (Fry, '28, Chart I.). .

The clear fixation of rays does not necessarily involve the presence of a definitely demarked centrosome (Figs. 12 and 23), although such is usually the case. The same situation holds true in cytasters (Fry, '28, Chart I., Type D1). Although centrosomes may occasionally be absent when rays are clearly fixed, they are never present unless rays are clearly fixed, and they are always absent when rays are fixed vaguely or not at all.

The different reagents produce a wide and significant diversity in the structure of centrosomes. They may be small and clearly delimited, whether granular (Fig. 4), or vacular (Fig. 10). They may be large and clearly delimited, whether granular (Figs. 2 and 26), or pleuricorpuscular (Fig. 27, etc.), or vacular (Fig. 6, etc.). They may be vaguely delimited, whether granular (Fig. 23, etc.), or vacular (Fig. 8, etc.). Various types of zoning occur about them, and there are other detailed modifications. If centrosomes have an actual existence in the living cell as individualized definitely-formed structures, it would be expected that most of their coagulation products would be somewhat similar to each other, as is the case with respect to the chromosomes. The extensive variability in centrosome structure caused by the fixatives, coupled with the fact that they occur only when rays are clearly coagulated, is further indication that they are nothing but coagulation products of the focal point of the rays.

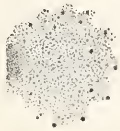
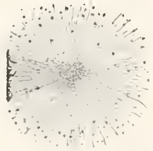
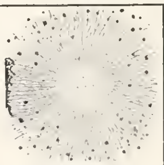
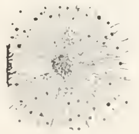
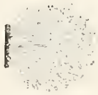
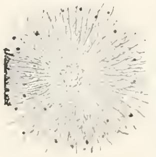
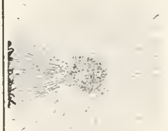
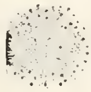
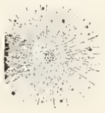
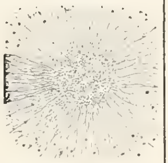
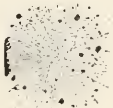
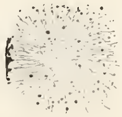
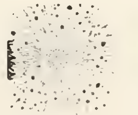
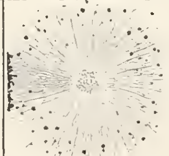
No correlation can be observed between the various types of ray configurations and the different classes of centrosomes. They may be definitely delimited and granular, whether rays are coarse (Fig. 16), or delicate (Fig. 4); or they may be vaguely delimited and vacular, whether rays are coarse (Fig. 15) or delicate (Fig. 8). The variations in centrosome structure are probably explained by the diverse chemical effects of the different reagents acting upon the material at the astral center, independently of whether or not the rays have a coarse or a delicate fixation.

Not only does the fixative modify the centrosome structure of metaphase asters, but it also modifies the exact time when centrosomes appear and disappear. Large numbers of asters in all stages from early prophase to late telophase were studied in the following reagents: corrosive sublimate, sublimate-acetic, absolute alcohol, absolute alcohol and acetic acid, Burkhardt's reagent, Benda's reagent, Flemming's fluid, Bouin's fixative, and a picro-acetic mixture. The results of this phase of the work are not illustrated. In those reagents where rays are clearly fixed and hence centrosomes occur, the exact time of their appearance and disappearance differs in the various fixatives. In some cases centrosomes arise during prophase, in others not until metaphase. The exact time when the enlarged central body (centrosphere) of the later stages disappears. also shows variability in the various reagents. This result is in harmony with the behavior of central bodies in cytasters (Fry, '28, Chart III.), where the time of their appearance and disappearance shows considerable variability in different fluids. Such differences in the time schedule are explained by the fact that a given reagent may cause the clear coagulation of rays during prophase, whereas another fluid does not accomplish this until metaphase. Hence the central bodies make their appearance at different stages, depending upon the fixative used. This is further evidence that central bodies are nothing but coagulation products of the focal point of clearly fixed rays, since they appear and disappear simultaneously with the appearance and disappearance of such rays.

FIX-ATIVE GROUP	RAYS NOT FIXED "CENTRAL BODIES" ABSENT	RAYS FIXED "CENTRAL BODIES" PRESENT		
I MERCURIC CHLORIDE	 MERCURIC CHLORIDE SAT. SOL. ["Corrosive Sublimate"] 1	 MERCURIC CHLOR. SAT. SOL. 99 VOLS. FORMIC ACID 1 VOL. 2  MERCURIC CHLOR. SAT. SOL. 99 VOLS. HYDROCHLORIC ACID 1 VOL. 5	 MERCURIC CHLOR. SAT. SOL. 99 VOLS. ACETIC ACID 1 VOL. ["Sublimate Acetic"] 3  MERCURIC CHLOR. SAT. SOL. 80 VOLS. ACETIC ACID 20 VOLS. ["Sublimate Acetic"] 6	 MERCURIC CHLOR SAT SOL. 99 VOLS. BUTYRIC ACID 1 VOL. 4
II CHLORO-FORM	 CHLOROFORM SATURATED SOL. 7	 CHLOROFORM SAT. SOL. 99 VOLS. ACETIC ACID 1 VOL. 8		
III ABSOLUTE ALCOHOL	 ABSOLUTE ALCOHOL 9	 ABSOLUTE ALCOHOL 99 VOLS. ACETIC ACID 1 VOL. 10	 ABSOLUTE ALCOHOL 75 VOLS. ACETIC ACID 25 VOLS. [Carnoy] 11	 ABSOLUTE ALCOHOL 300 VOLS. DISTILLED WATER 200 VOLS. ACETIC 90 VOLS. - NITRIC 10 VOLS. MERCURIC CHLORIDE TO SATURA. [Petrunkewitsch] 12

THE EFFECTS OF VARIOUS FIXATIVES UPON THE AND CENTROSOMES IN METAPHASE ASTERS

Asters are in the metaphase ("late") stage. They were similar in structure in the living eggs. The present differences are due to the various coagulation effects of different fixatives. In each figure each dimension and the physical structure of each part is an average of the observations made in a number of asters, studied with reference to the points listed in Table I., p. 135. The chromosomes, however, were studied only superficially. The figures are 650 × enlarged. The diameter of the aster in Fig. 3, for example, is 28 μ. Asters are grouped horizontally with reference to classes

FIXATIVE GROUP	RAYS NOT FIXED "CENTRAL BODIES" ABSENT	RAYS FIXED "CENTRAL BODIES" PRESENT		
IV POTASSIUM DICHROMATE CHROMIC ACID	 POTASSIUM DICHROMATE 1% 13	 CHROMIC ACID 1% 14	 POTASS. DICHROM. 5% 30 vols. CHROMIC ACID 1% 60 vols. ACETIC ACID 5 vols. [Burckhardt] 15	 POTASSIUM DICHROM. 2.5 gms. SODIUM SULPHATE 1 gm. MERCURIC CHLORIDE 5 gms. ACETIC 5 c.c. - WATER 100 c.c. [Zenker] 16
V OSMIC ACID	 OSMIC ACID 2% [Kopsch] 17	 OSMIC ACID 2% 4 vols. CHROMIC ACID 1% 15 vols. DISTILLED WATER 19 vols. [Benda] ["Flemming without acetic"] 18	 OSMIC ACID 2% 4 vols. CHROMIC ACID 1% 15 vols. ACETIC ACID 1 vol. DISTILLED WATER 20 vols. [Weak Flemming] 19	 OSMIC ACID 2% 4 vols. PLATINIC CHLORIDE 1% 15 vols. ACETIC ACID 1 vol. [Hermann] 20
VI FORMOL [NEUTRAL]	 FORMOL [NEUT. WITH $MgCO_3$] 10% 21	 FORMOL [ACID] 20 vols. POTASS. DICHROM. 3% 80 vols. [Regaud] 22	 FORMOL [ACID] 20 vols. POTASS. DICHROM. 3% 80 vols. ACETIC ACID 1 vol. [Regaud] 23	
VII FORMOL [ACID] ACETIC ACID PICRIC ACID	 FORMOL [ACID] 10% 24	 ACETIC ACID 1% 25	 PICRIC ACID SAT. SOL. 26	 FORMOL [ACID] 25 vols. PICRIC ACID SAT. SOL. 75 vols. ACETIC ACID 5 vols. [Bouin] 27

RELATIONSHIP BETWEEN CLEARLY FIXED RAYS OF FERTILIZED *ECHINARACHNIUS* EGGS

of fixatives: they are grouped vertically with reference to whether or not rays are clearly fixed. Central bodies (centrosomes) are present only in those fixatives that clearly coagulate rays; they are absent when rays are not clearly fixed, even though in such cases the spindle fibers may be coagulated. The very wide diversity in centrosome structure is significant. These facts indicate that the so-called central bodies (centrosomes) of *Echinarachnius* are nothing but coagulation artifacts of the focal point of clearly fixed rays.

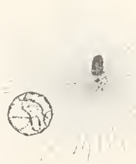
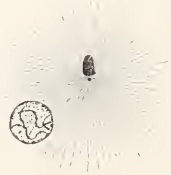
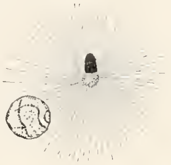
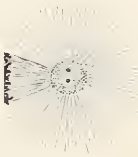
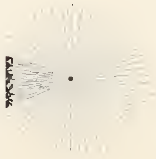
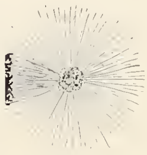
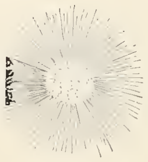
It has been suggested that central bodies may have the same chemical composition as that of rays, hence a fixative that fails to clearly coagulate rays will therefore probably fail to show central bodies. The answer to this is as follows. In the previous study (Fry, '29), during telophase stages (Figs. 25 and 26), rays are fixed very clearly in the outer part of the aster whereas they are vague or absent in the central area. These asters have no centrosomes, although the fixative is capable of clearly coagulating rays in the peripheral part of the asters, and also shows centrosomes in the earlier stages where clear rays reach the center. Similarly, in the study of cytasters (Fry, '28) there is a large number of asters (Types C1 and C2) without central bodies, where rays are clearly fixed peripherally but are absent centrally. If central bodies have the same chemical composition as the rays they should be demonstrated in such cases, since the fixative is capable of clearly fixing rays in the outer part of the asters. Another source of evidence, in this connection, is found in three instances (Figs. 1, 17 and 24) in which the spindle fibers are quite clearly coagulated, whereas the rays are practically unfixed and centrosomes are absent. Since the fixative is able to coagulate the spindle fibers in these cases, and since it coagulates the rays very vaguely in two of them (Figs. 1 and 24), it should also show centrosomes assuming that the latter have the same chemical composition as rays and spindle fibers. Such anastral figures, produced by the fixative, in which the centrosome is absent, indicate that centrosomes are present only when clearly fixed astral rays converge from all sides; the clear fixation of the spindle fibers without such fixation of rays does not produce centrosomes.

It has also been suggested that only those fixatives that penetrate rapidly may give an accurate coagulation picture of the living condition, and that they alone may be capable of clearly fixing rays and of showing the central body; whereas, fixatives that penetrate slowly may cause a partial break down of the cell structures so that rays are unfixed and the central body is not shown. The answer to this suggestion is as follows. Aside from the fact that data are unavailable concerning which reagents penetrate rapidly or slowly, it is not necessarily a valid assumption that a rapidly penetrating fixative gives a coagulation product more like the living condition than one that penetrates slowly. The concepts "slow" and "rapid" when applied to molecular dimensions within the protoplasm are of doubtful value with reference to the validity of coagulation products.

Thus, whether the coagulated ray structure of fertilization asters in *Echinarachnius* eggs is modified by various fixatives (the subject of the present paper), or by various stages in the astral history (Fry, '29), or whether similar modifications are produced in cytasters of artificially activated eggs (Fry, '28), the same conclusion holds good. The so-called centrosomes of *Echinarachnius* eggs are coagulation artifacts of the substance at the astral center, produced by fixation only when rays are clearly coagulated and reach the mid-point; they have no existence as individualized structures in the living condition.

The Status of Echinoderm Central Bodies in the Light of the Present Investigation.

The chart presenting Figures 28 to 34 (p. 144) constitutes a résumé of former studies of central bodies in echinoderm fertilization (cf. Fry, '29, pp. 105–109; 120–121). Both in sperm-asters and in cleavage-asters earlier investigators describe the same three classes of central bodies: (1) a type composed of a minute period-like granule, the centriole, surrounded by a larger diffuse structure, the centrosome; (2) a type composed of a minute centriole only, without a centrosome; (3) a type composed only of the larger structure, the centrosome, without a centriole. In this classification the terms "centriole" and "centrosome" are used in the same manner as previously discussed (p. 132). Underneath each figure is given a list of the species which have been described as possessing that type of central body, together with the investigators reporting the observations. No attempt has been made to make the lists complete but they include the major papers in the field. Although the drawings of the various classes are in each case a diagrammatic presentation of several original figures made by different workers to illustrate the situation in various species, they are, nevertheless, accurate delineations of the major details of the originals.

CENTRIOLE AND CENTROSOME	CENTRIOLE ONLY	CENTROSOME ONLY	
IN SPERM ASTERS			
 Fig. 28	 29	 30	
Echinus (Boveri,'00) Sphaerechinus (Hill,'95 and Erlanger,'98)	Echinus (Kostanecki,'96) Asterias (Wilson,'23) Toxopneustes, (Wilson,'00, '28)	Echinus (Ziegler,'04) Arbacia, Asterias (Wilson and Mathews,'95) Toxopneustes (Wilson,'95,'96)	
IN CLEAVAGE (METAPHASE) ASTERS			
		PLEURICORPUSCULAR TYPE	VACULAR TYPE
 31	 32	 33	 34
Echinus (Boveri,'00) Sphaerechinus (Hill,'95; and Erlanger,'98)	Echinus (Kostanecki,'96)	Sphaerechinus (Bütschli,'92) Toxopneustes (Wilson,'00,'28) Arbacia (Wilson & Mathews,'95)	Echinus (Ziegler,'04) Toxopneustes (Wilson,'95,'96) Parechinus (Meves,'12)

TYPES OF CENTRAL BODIES PREVIOUSLY DESCRIBED IN FERTILIZED ECHINODERM EGGS

The drawings diagrammatically illustrate central body structures previously described in echinoderm fertilization. There are three classes: (1) those composed of a centriole and a centrosome; (2) those composed of a centriole only; (3) those composed of a centrosome only. The upper figures illustrate the situation in sperm-asters; the lower ones show cleavage (metaphase) asters. Underneath each illustration is a list of the species having that class of central bodies, together with the investigators reporting the observations. The present study indicates that previously described echinoderm "centrosomes" are actually coagulation artifacts of the focal point of clearly fixed rays, and that "centrioles" are actually cytoplasmic granules that occasionally occur at the mid-point of the asters.

It will be observed that a minute period-like type of central body, is described by many of the investigators. This fact supports the assumption that in many cases the central body in echinoderm fertilization is a typical centriole similar to the structure supposed to occur generally in animal cells. On the other hand, it is apparent that the previous studies show a considerable diversity in central body structure. In *Echinus*, for example, in both sperm- and cleavage-asters, all three types of central bodies are reported by various workers. Similarly, the phenomena in *Asterias, Toxopneustes* and *Sphaerechinus* are variously described. The causes of these discrepancies are explained by the present study.

The central body type composed of both centriole and centrosome (Fig. 31) is described most fully by Boveri ('00) in *Echinus*. He used a picro-acetic fixation composed of one part glacial acetic acid, and ninety nine parts of a saturated solution of picric acid diluted with two volumes of water. At Boveri's suggestion, Coe investigated the effects of various fixatives upon sea urchin eggs. Boveri states: "the sections prove that none of the mediums used surpass the picro-acetic mixture; the best of them does not even approach it" ('00, p. 30). The central body structure he describes in metaphase asters of *Echinus* eggs produced by his picro-acetic fixative (Fig. 31; cf. Fry, '29, Figs. 7–10), is very similar to that occurring in *Echinarachnius* eggs when similarly fixed. In both species there is a large, homogeneously granular centrosome. Furthermore, if the situation in *Echinus* is similar to that in *Echinarachnius*, the supposed centrioles illustrated by Boveri, are actually cytoplasmic granules that happen to occur at the astral center. In the present investigation the cytoplasmic granules were carefully studied, and there is wide variety in this respect. In the case of some fixatives such granules are absent (Fig. 18, etc.); in others they are small, whether few (Fig. 23, etc.), or numerous (Fig. 14, etc.); in others they are large, whether few (Fig. 24, etc.), or numerous (Fig. 26, etc.). In the case of picro-acetic fixation the granules are very numerous. In all fixatives they tend to be excluded from the central portion of asters, and are abundant in the outer parts; they do occur sporadically, however, in the central areas. The more abundant they are in the cytoplasm generally, the more apt are they to be found at the astral center, hence fixation with picro-acetic favors their occasional occurrence near the mid-point. A study of *Echinarachnius* metaphase figures, fixed in picro-acetic, shows that most of the asters contain no granules; a few have one, or two, or three, or more. The granules vary as to number, as to size, and as to location in the "centrosome"; the situation concerning granules in one aster of a metaphase figure, gives no clue whatever concerning the condition existing in the other aster. Hence in *Echinarachnius* eggs, configurations occur in a very limited number that exactly duplicate Boveri's illustrations of *Echinus* central body structure, having one or two "centrioles" surrounded by a "centrosome.' Such configurations, however, are without any significance. It will require a study of *Echinus* eggs by the methods of the present investigation to prove whether or not the condition existing in *Echinarachnius* applies to *Echinus*. It is highly probable, however, that the factors mentioned above, *i.e.*, coagulation artifacts of the astral center, and the random occurrence of cytoplasmic granules, explain not only Boveri's observations but also the other types of echinoderm central bodies formerly illustrated.

That type of central body, previously described, that is composed of a minute centriole only (Fig. 32), is probably explained by a misinterpretation of a cytoplasmic granule occurring at the center of an aster, that is fixed by a reagent which does not produce a demarked centrosome.

The type of central body composed of a large "centrosome" only, without a centriole, occurs in two major classes, each of which shows many modifications: the first type is definitely delimited, having a mulberry-like or pleuricorpuscular structure, and is darkly stained (Fig. 33); the second type is larger and less clearly delimited, having a vacular or reticular structure, and is lightly stained (Fig. 34). The center of the latter type is in some cases practically empty as though the substance had been dissolved away. These two classes are well illustrated in Wilson's study of *Toxopneustes*. In his earlier work ('95) he uses eighty parts of a saturated solution of mercuric chloride, with twenty parts of glacial acetic acid. In his later work ('00) a sublimate-acetic mixture is again used, but in this case there is a smaller percentage of acetic acid.[6] It is observed that the larger quantity of acetic acid produces the large, lightly-stained vacular "centrosome" (Fig. 34); whereas the lesser amount produces the dark, small pleuricorpuscular type (Fig. 33). This situation is exactly duplicated in the case of *Echinarachnius*, where a sublimate-acetic mixture containing twenty per cent. acetic acid produces a light vacular center (Fig. 6), whereas one containing but one per cent. acetic acid produces a dark pleuricorpuscular center (Fig. 3). This case is but another illustration of the ability of the fixing agent to modify the coagulated central body structure.

Thus it appears that previous studies of echinoderm central bodies are probably explained by the present investigation, although the possibility of the effect of species differences must be kept in mind. When the various types of central bodies formerly described (Figs. 28–34) are analyzed in the light of the present study, it seems highly probable that the phenomena are due to two fallacies: first, the so-called centrosomes are actually coagulation products of the focal point of well formed rays; second, the so-called centrioles are actually cytoplasmic granules that occur sporadically at the astral center.[7] This situation completely invalidates the usual hypotheses concerning central bodies in echinoderms, a review of which has been given previously (Fry, '29, pp. 101–104). *If future study of echinoderm central bodies in various species shows that the phenomena are like those in Echinarachnius, it will prove, at least in this phylum, that the current central body hypotheses are actually based upon misinterpretations of coagulation artifacts and cytoplasmic granules.* The reasons have been given previously (Fry, '29, pp. 121–123) why it is probable that this conclusion may be found to have wide application to central bodies in fertilization and mitosis generally.

The wide differences between the conclusion of former investigations and that of the present study concerning the significance of echinoderm "central bodies," is explained by the equally wide difference between the method of study used by former investigators and that of the present work (Fry, '28, pp. 387–392; '29, pp. 109–113). The usual cytological procedure is to use a limited number of fixatives and to select certain of the coagulation products as "normal," dismissing the others as "poorly fixed," *without a sufficiently complete and quantitative consideration of all possible classes of coagulation products.* Both methods have the same aim, that of determining which coagulation products are "normal," *i.e.,* which of them most nearly approximate the living condition. The methods differ concerning the completeness of the analysis necessary to decide what is "normal." The old method is open to the danger that the "normal" is determined upon the basis of incomplete and partial data. The present method makes a more complete analysis. It is not a superficial study of many classes of coagulation products, in contrast to an intensive study of a few "best" types; it is an equally intensive study of all types, omitting none, in order to determine what is "normal."

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[6] This sublimate-acetic mixture contained one or two per cent. glacial acetic acid according to information received from Prof. Wilson.

[7] A third source of error in early sperm-asters is a misinterpretation (Boveri, '00) of the bi-lobed granule of the sperm's middle-piece (cf. Fry, '29, p. 105).

The prophecy is made that if future study of cellular components is carried on by the simple quantitative method used in the present work, that various cytological facts, now assumed to be based on sound evidence, will be found actually to be based upon certain selected groups of coagulation products, without a sufficient consideration of their quantitative relationships in relation to other classes. Results may possibly be attained in various fields, as significant as are those here reported concerning the so-called central bodies in echinoderm fertilization.

RÉSUMÉ.

1. When metaphase asters of the first cleavage division in *Echinarachnius* eggs are fixed in a wide variety of reagents, central bodies (centrosomes) are present only in those cases where the fixative clearly coagulates rays. They are absent when astral rays are fixed vaguely or not at all.

2. This result is in harmony with that of the preceding studies (Fry, '28, '29). Hence whether astral structure is modified by the various stages in the normal mitotic cycle, or by various fixatives: whether this occurs in nuclear asters of fertilized eggs or in cytasters of artificially activated eggs; central bodies (centrosomes) are present only when clearly fixed rays reach the astral

center. This indicates that they are nothing but coagulation artifacts of the focal point of well formed rays and that they have no existence as individualized bodies in the living condition.

3. It is probable that the phenomena in *Echinarachnius* are typical of echinoderms generally. The illustrations in previous studies of echinoderm central bodies fall into three classes: (1) those composed of a centriole which is a minute period-like granule, surrounded by a centrosome which is a larger, more diffuse structure; (2) those composed of the minute centriole only; (3) those composed of the larger centrosome only. The present investigation indicates that the supposed "centrosomes" are actually coagulation artifacts of the focal point of well formed rays, and that the supposed "centrioles" are actually cytoplasmic granules that have a random occurrence at the mid-point of asters. These facts completely invalidate the usual hypotheses concerning central bodies in echinoderms.

4. It is probable that this conclusion may have wide application to central bodies in fertilization and mitosis generally. Whether or not such is the case requires further study of various species in the various phyla, by the same quantitative method used in the present investigation.

LITERATURE CITED.

Boveri, T.

'00 Zellen-Studien. Heft 4. Ueber die Natur der Centrosomen. Jen. Zeitsch., 25.

Bütschli, O.

'92 Untersuchungen über microscopische Schäume und das Protoplasma. English translation by E. A. Minchin. Adam and Charles Black, London.

Erlanger, von R.

'98 Zur Kenntnis der Zell- und Kernteilung. II. Ueber die Befruchtung und erste Teilung des Seeigel-Eies. Biol. Cent., 18.

Fry, H. J.

'28 Conditions Determining the Origin and Behavior of Central Bodies in Cytasters of *Echinarachnius* Eggs. BIOL. BULL., 54.

'29 The So-called Central Bodies in Fertilized *Echinarachnius* Eggs. I. The Relationship between Central Bodies and Astral Structure as Modified by Various Mitotic Phases. BIOL. BULL., 56.

Hill, M. D.

'95 Notes on the fecundation of the egg of *Sphærechinus granularis* and on the maturation of the egg of *Phallusia mammilata*. Quart. Journ., 38.

Kostanecki, K.
'96 Ueber die Gestalt der Centrosomen im befruchteten Seeigelei. Anat. Hefte, 22.
Meves, R.
'12 Verfolgung des sogenannten Mittelstückes des Echinidenspermiums im befruchteten Ei bis zum Ende der ersten Furchungsteilung. Arch. mikr. Anat., 80.
Wilson, E. B.
'95 Archoplasm, Centrosome and Chromatin in the Sea-urchin Egg. Jour. Morph., 11.
'96 The Cell in Development and Inheritance, first edition. The Macmillan Co., New York.
'00 The Cell in Development and Heredity, second edition. The Macmillan Co., New York.
'23 The Physical Basis of Life. Yale University Press.
'28 The Cell in Development and Heredity, third edition, 1925, with corrections. The Macmillan Co., New York.
Wilson, E. B., and Mathews, A. P.
'95 Maturation, Fertilization and Polarity in the Echinoderm Egg. Jour. Morph., 10.
Ziegler, H. E.
'04 Die ersten Entwickelungsvorgänge des Echinodermeneies insbesondere die Vorgänge am Zellkörper. Denkschrift med.-naturw. Gesell. Jena (Festschrift z. E. Haeckel).

THE SO-CALLED CENTRAL BODIES IN FERTILIZED *ECHINARACHNIUS* EGGS.

III. The Relationship between Central Bodies and Astral Structure as Modified by Temperature.[1]

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Washington Square College, New York University.

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Introduction.

Two previous studies have demonstrated that the supposed central bodies in fertilized *Echinarachnius* eggs are actually nothing but coagulation artifacts of the focal point of clearly fixed rays, misinterpreted as "centrosomes," plus the occurrence of cytoplasmic granules at the astral center, misinterpreted as "centrioles." This conclusion renders meaningless the usual hypotheses concerning echinoderm central bodies, *i.e.*, that they are individualized structures which are the formative foci of asters, and that they may have genetic continuity from cell to cell and from generation to generation.[2]

In the first of these studies (Fry, '29*a*) the fixative and the environment are constant. Modifications in astral structure occur during different stages in the normal mitotic cycle: centrosomes are present only during the middle history of sperm- and cleavage-asters when rays are clear; they are absent at other times when rays are vague. In the second study (Fry, '29*b*) the astral stage (metaphase) and the environment are constant. Metaphase astral structure is modified by the different coagulation effects of various fixatives: centrosomes are present only in the case of those reagents that clearly coagulate rays; they are absent when rays are fixed vaguely or not at all. In the present study the astral stage and the fixative are constant. Astral structure is modified by changes in the environment with reference to temperature.

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[1] The material was secured at the Marine Biological Laboratory, Woods Hole, Massachusetts.

[2] A review of the literature concerning central bodies in echinoderm fertilization, together with a brief discussion of terminology, will be found in the earlier papers of this group (Fry, '29*a*, pp. 101–109; Fry, '29*b*, p. 132 and pp. 143–147).

If the conclusion established by the previous studies is correct, that *Echinarachnius* central bodies (centrosomes) are actually coagulation artifacts of the inner ends of clearly fixed rays, then in the present study they should occur only at those temperatures at which rays are well formed in the living condition, and are clearly fixed when a proper reagent is used: they should be absent at those temperatures when rays are vaguely formed in the living egg, and hence are vaguely fixed.

The various sets of eggs were fertilized at 18° C. Within a minute or so after insemination each set was transferred to sea water of a given temperature. Sets were run at two degree intervals from 4° C. to 28° C. There is a very low percentage of development at 4° C., hence there are no experiments at lower temperatures. Eggs cytolyze at 28° C., hence this is the upper temperature limit. The temperature of each set did not fluctuate more than two tenths of a degree.

Eggs were fixed in a sublimate-acetic mixture (saturated solution of mercuric chloride, 97.5 per cent.; glacial acetic acid, 2.5 per cent.). This fixative is commonly used in the cytological study of echinoderm eggs. It is a reagent that coagulates rays clearly. Eggs were sectioned at 5 μ thick and stained with Heidenhain's haematoxylin. They were studied at 750 × magnification.

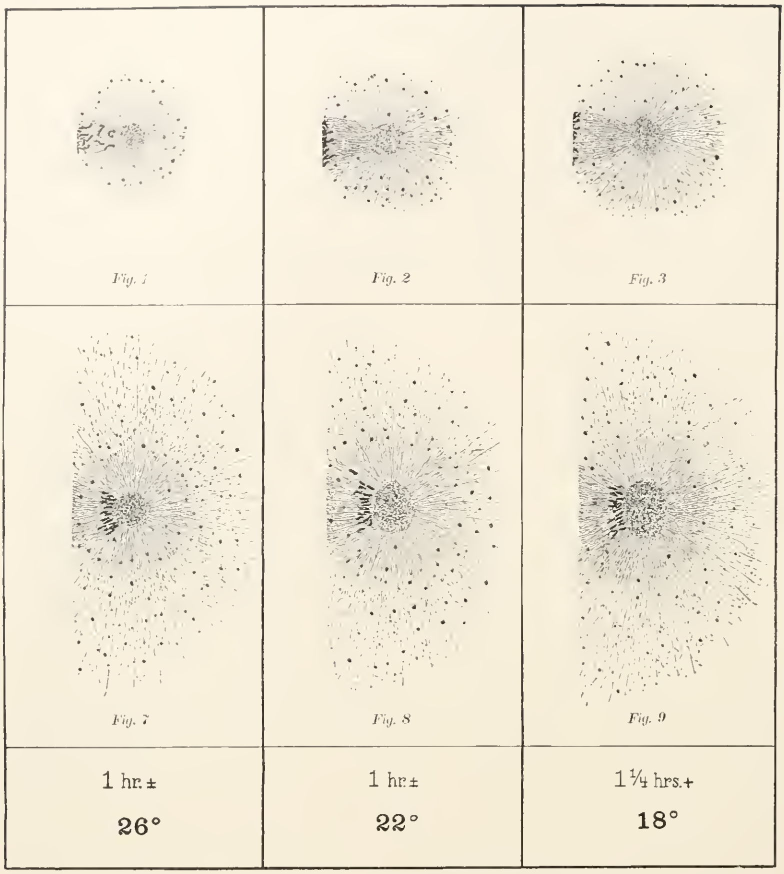
At each two degree temperature interval, about twenty-five metaphase and about twenty-five anaphase figures were each measured and analyzed with reference to the twenty-two points previously listed for the study of cleavage asters (Fry, '29*b,* Table I., p. 135). Only such eggs were selected which happen to be sectioned in a plane more or less parallel to that of the elongation of the mitotic figure. In the case of the metaphase asters, only those were studied that have rays of maximum clarity, and the chromosomes aligned in two groups ready to separate (*i.e.*, "late" metaphase asters), in contrast to a slightly earlier stage in which the rays are less clear and the chromosomes are in a tangled mass (*i.e.*, "early" metaphase asters). In the case of anaphase figures, only those were studied in which the chromosomes have passed to the end of the spindle but still maintain the typical elongate form, in contrast to a slightly later stage when they exist as rounded vesicles.

The figures (pp. 154–155) are arranged in vertical columns, each of which illustrates the metaphase and anaphase condition at a given temperature. Although the material was fixed and studied at two degree intervals, the illustrations show the phenomena only at four degree intervals, since such procedure adequately presents the facts. In the vertical column for each temperature, the metaphase aster is shown above, with the anaphase aster below it. For each temperature is given the approximate time after fertilization required for the eggs to reach that stage when metaphase and anaphase figures are most numerous.

The illustrations are 650 × enlarged. For example, the coagulated metaphase aster at 18° C. (Fig. 3) is $32\,\mu$ in diameter; the dimensions of the anaphase aster at the same temperature (Fig. 9) are $70\,\mu \times 35\,\mu$. In each figure, every dimension and the physical appearance of each part is an average of all the observations made in about twenty-five asters belonging to that class. The chromosomes, however, are an exception to this; they were studied only superficially. The method of study used in the present work is the same as that previously described (Fry, '28, pp. 387–392; '29*a*, pp. 109–113).

THE EFFECTS OF VARIOUS TEMPERATURES UPON THE RELATIONSHIP BETWEEN CLEARLY FIXED RAYS AND CENTRAL BODIES IN CLEAVAGE ASTERS OF FERTILIZED *ECHINARACHNIUS* EGGS.

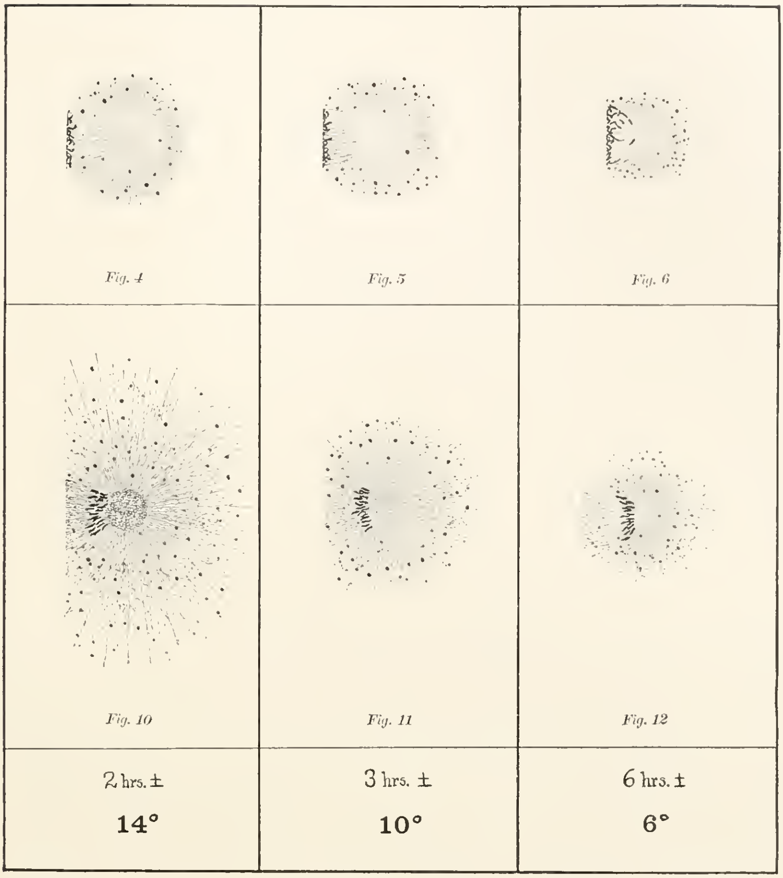
A study of the figures shows that in metaphase asters a well formed pleuricorpuscular central body (centrosome) is present only at temperatures from 26° C. to 18° C., when rays are clear



THE EFFECTS OF VARIOUS TEMPERATURES UPON THE CENTROSOMES IN CLEAVAGE ASTERS

The figures are arranged in vertical columns with reference to four degree temperature intervals. Metaphase asters are above; anaphase asters are below. In each figure each dimension and the physical structure of each part, is an average of the observations made in about twenty-five division figures, according to the points listed, Fry, '29*b*, Table I., p. 135. The chromosomes, however, were studied only superficially. The figures are

(Figs. 1–3). The rays begin to fade at 14° C., and the central body is much less definite (Fig. 4). From 10° C. to 6° C. the rays are very vague and there is no central body (Figs. 5–6). The same situation holds in anaphase asters with the exception that the rays fade at a somewhat lower temperature than is the



RELATIONSHIP BETWEEN CLEARLY FIXED RAYS AND OF FERTILIZED *ECHINARACHNIUS* EGGS.

650 × enlarged. The fixative is a saturated solution of mercuric chloride, 97.5 per cent., plus glacial acetic acid, 2.5 per cent. Central bodies (centrosomes) are present at the upper temperatures where rays are clear. They fade out as the rays become vague at the lower temperatures. This indicates that the so-called centrosomes are coagulation artifacts of the focal point of clearly fixed rays. There are no centrioles.

case in the metaphase stage. In both cases the central bodies disappear simultaneously with the fading of clear rays. This result is in harmony with that obtained in a study of the effect of various environmental factors upon cytasters in artificially activated eggs (Fry, '28, Chart II.), where central bodies occur only when the environmental factors produce cytasters with well formed rays.

This study yields further evidence supporting the conclusion that the so-called central bodies (centrosomes) of *Echinarachnius* eggs are coagulation artifacts. No matter how the ray structure of coagulated asters is modified, whether by the astral stage, or by fixation, or by environmental agents, centrosomes occur only when rays are clearly fixed and reach the center.

There is no evidence for the presence of centrioles. As in the previous studies, cytoplasmic granules are abundant throughout the cytoplasm. They are numerous among the outer portion of the rays, but occur only sporadically in the centrosome, differing as to number, size, and location. A very limited percentage of such granule configurations simulate centrioles, but they are without significance.

Observations Concerning the Spindle.

The method of study here used concerning the central body problem also yields significant facts concerning the asters and spindles, as well as the interrelations of these components of the achromatic part of the mitotic mechanism.

This investigation yields data concerning the rôle of the spindle in contrast to that of the asters. Keeping in mind the fact that but one fixative has been used, it is observed in metaphase asters that at 26° C. to 18° C. (Figs. 1–3) the spindle fibers are very numerous and lie close together, whereas at 14° C. to 6° C. (Figs. 4–6) they are less numerous and lie farther apart. Nevertheless, at the lower temperatures, the spindle maintains its identity in a manner not shared by the asters. At 6° C. (Fig. 6) the metaphase aster is very small, and is composed of exceedingly vague rays in contrast to the large clear-rayed aster occurring at 18° C. (Fig. 3); whereas at 6° C. the spindle maintains the same width and roughly occupies the same area as it does at the higher temperatures. It seems, therefore, that the spindle is that part of the achromatic mechanism least effected by temperature changes. The significance of this fact requires further study.

It is to be remembered that the present paper is an analysis of the effects of temperature upon only a small part of the astral cycle, when using but one fixative. It is necessary to supplement this work by a similar study of the mitotic figure at all stages from fertilization to first cleavage; to duplicate the method of study in a variety of fixatives; and to consider the cytological observations with reference to percentages of normal and abnormal development occurring at each temperature. Such a procedure may yield valuable data concerning the division figure.

The previous study of the effect of fixatives (Fry, '29*b*) yields facts concerning the structure of the spindle. With the exception of several coagulation products in which neither the asters nor the spindle are fixed (Figs. 7, 9, 13 and 21), the spindle shows a structure more definitely fibrous than that of the astral rays. In cases where the rays are coarsely fixed, the spindle fibers are even more definite; in cases where the rays are delicately fixed the spindle fibers stand out far more distinctly; in cases where the rays are practically unfixed (Figs. 1, 17 and 24) the spindle fibers are quite clear. It is well known that in the living condition the spindle generally appears to be structureless, whereas the aster shows a radial arrangement. Since in practically all fixatives the spindle appears to be more definitely fibrous than the ray configuration of the aster, this indicates that such is actually the case in the living condition. Such a definite linear organization can scarcely be regarded as a coagulation artifact since it occurs in so many diverse fixatives. This appears to be an instance where the fixed material yields data more dependable than does a study of the living condition.

RÉSUMÉ.

1. When fertilized *Echinarachnius* eggs are allowed to develop at various temperatures, and are fixed in a sublimate-acetic mixture, central bodies (centrosomes) are present in metaphase and anaphase asters only at the upper temperature intervals where rays are well formed. Simultaneously with the fading of the rays at low temperatures, the centrosomes disappear.

2. This result is in harmony with that of the previous studies of this group. Whether the structure of fertilization asters, or of cytasters, is modified by the various stages in the normal mitotic cycle, or by fixation, or by modifications of the environment,

centrosomes are present only when clearly fixed rays reach the astral center. They are therefore nothing but coagulation artifacts of the focal point of well formed rays, having no existence as individualized bodies in the living condition.

3. There is no evidence for the presence of centrioles. They are occasionally simulated, however, by cytoplasmic granules.

4. The method of study used in this group of papers also yields significant data concerning the mitotic mechanism as a whole. The spindle has a structure more clearly fibrous than is the ray configuration of the asters. Under the influence of low temperatures the spindle maintains its typical dimensions in contrast to the behavior of the asters which almost disappear.

LITERATURE CITED.

A bibliography of the literature concerning central bodies in echinoderm fertilization will be found in the first and second papers of this group (Fry, '29*a*; '29*b*). A list of those papers belonging to the present series of studies follows:

Fry, H. J.

'28 Conditions Determining the Origin and Behavior of Central Bodies in Cytasters of *Echinarachnius* Eggs. Biol. Bull., 54.

'29*a* The So-called Central Bodies in Fertilized *Echinarachnius* Eggs. I. The Relationship between Central Bodies and Astral Structure as Modified by Various Mitotic Phases. Biol. Bull., 56.

'29*b* The So-called Central Bodies in Fertilized *Echinarachnius* Eggs. II. The Relationship between Central Bodies and Astral Structure as Modified by Various Fixatives. Biol. Bull., 57.

CLEAVAGE RATES IN FRAGMENTS OF CENTRIFUGED *ARBACIA* EGGS.

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In order to test whether the granules in the sea urchin egg affect the rate of cleavage, *Arbacia* eggs were centrifuged, and before appreciable redistribution of the stratified materials could take place, were cut along the plane of stratification into two approximately equal halves. One half was practically free from granules, the other contained practically all of them. The two halves of each egg were subjected to the same degree of injury in cutting, since they were the result of one operation, and they were then placed together in a small glass dish in a moist chamber, and were inseminated.

The eggs of *Arbacia* contain an abundance of granular material, especially pigment, and they can readily be stratified with the centrifuge. The two halves of the centrifuged egg differ markedly in viscosity as well as in granular content. The clear half is considerably less viscious than the normal egg, and the granular half is considerably more so.

On account of the difficulties of operation, and of the high mortality, especially of fragments of centrifuged eggs, together with limited time in which to work, not enough cases of all sorts were obtained to make the actual average times recorded in the results highly significant, but certain relationships are significantly revealed.

Method.

Stratifying and Cutting.

Arbacia eggs are completely stratified by centrifuging for twelve minutes at about 1,350 gravities (2,600 r.p.m. with 18½ cm. radius). The materials of the egg which are moved by the centrifuge come to lie in four sharply defined specific gravity zones. The smallest and lightest of these is a small oil cap composed of comparatively large droplets. The next zone is clear and supposedly watery. It is seen by oil immersion lens and dark field illumination to contain very few particles which are not colloidal, or nearly so. The egg nucleus lies in this watery zone just under the oil cap. The third and largest zone contains the bulk of the granular material of the egg including the yolk and the finer particles of pigment. The fourth and densest zone contains most of the pigment collected in a cap which is less sharply set off from the third zone than are the other zones from each other.

When the egg is completely stratified the plane of contact of the watery zone and the large granular zone passes near the equator of the egg but slightly toward the oil cap pole, so that the granular part comprises a little more than half of the egg. By means of a Taylor micro-manipulator eggs were cut into two fragments of approximately equal size, by two types of cuts: (1) Some were cut along the equator of the egg in which case the fragments were of equal size but the " clear " fragment contained a slight amount of granular material, and (2) others were cut along the plane of contact of the zones in which case the "clear" fragment was slightly smaller than the " granular." Fragments from these two types of cuts are grouped indifferently together in Table I. because no difference was found in the result.

In all cases the eggs were cut within twenty minutes after centrifuging. In uncut eggs redistribution of the stratified materials can be seen to have started half an hour after centrifuging.

After cutting, the fragments were not inseminated until ten minutes had passed. If then moderately weak sperm suspensions were used, polyspermy was almost entirely avoided. If the fragments were inseminated immediately after cutting polyspermy occurred oftener than not. The ten minute interval after cutting apparently allows the membrane to recover and repair and like the normal egg surface it becomes impervious to sperm after fertilization.

Nuclear Content.

Since the egg nucleus always lies in the watery zone of the centrifuged egg, it is always contained in the fragment which we refer to as " clear." Consequently, after fertilization the " clear " fragment is diploid and the "granular " is haploid.[1] It is known that haploid and diploid fragments of normal non-centrifuged Echinoderm eggs cleave at different rates. (Tennent, Taylor, Whitaker, 1929.) The diploid fragments cleave first. As a control, the time interval from insemination until the first cleavage of nucleated and non-nucleated fragments of normal non-centrifuged eggs was first measured. Eggs from the same female, and at the same temperature, were stratified and cut, so that on the difference of "haploid" and "diploid" the difference of "clear" and "granular" was superimposed. The added effect of this second condition was taken as the measure of the difference in cleavage rate due to approximately double the normal concentration of granules in one fragment and to an almost complete absence of granules from the other. A significant measure is furnished by a comparison of the difference in cleavage rate of haploid and diploid fragments of non-centrifuged eggs with the difference between haploid ("granular") and diploid ("clear") fragments of centrifuged eggs.

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[1] At least in the early cleavage stages.

Temperature.

The temperature for a particular experiment was approximately constant. Moist chambers were filled from the main salt water supply of the laboratory and were set aside together. After equilibrium had been reached with room temperature (as indicated by a thermometer) the temperature was recorded. It was usually slightly lower than room temperature due to a certain amount of evaporation from the outside of the glassware. During the course of the experiment, which lasted from forty minutes to an hour and a quarter, the temperature did not vary more than ±¼° C. But the two halves of the same egg, being compared for cleavage rate, were kept much closer yet to the same temperature as they were in the same dish of water, lying within a millimeter of each other, from the time of insemination until both fragments cleaved. The room temperature varied at different times and on different days so that the water temperatures ranged between 20° and 24½° as indicated in Table I.

Condition of Material.

The experiments were performed during the last two weeks of July, 1928. Eggs and sperm were in excellent condition. In all, the eggs of twenty different females were used; but many more females were discarded after preliminary tests. For it was found that even among eggs of which more than 95 per cent. develop good fertilization membranes, there is great variation in aptitude to cutting. This is especially marked after centrifuging. The eggs of some females tend to burst with the slightest provocation, while those of others can be cut with the greatest ease. For the present purpose it was of great importance to minimize injury to the fragments. This was largely realized by taking care in selecting suitable eggs, and in avoiding pressure and squeezing, and by discarding fragments known to be damaged.

RESULTS.

Explanation of Table I.

In the following table each different female has a number which for convenience has been assigned in the order of temperature sequence. The temperature indicated for each female applies within the limits stated, to all eggs taken from her: controls, centrifuged controls, and fragments of both non-centrifuged and centrifuged eggs. Time is counted to the nearest half minute. In the control dishes of both the centrifuged and non-centrifuged eggs time is counted from insemination until fifty per cent. of the eggs in the microscope field (usually about twenty-five) have just cleaved. But in the case of the fragments, the recorded time is the observed time for each particular fragment. A certain stage which could be repeatedly recognized was arbitrarily chosen and counted as the time of cleavage. This stage occurs just as the furrow has passed completely through the egg, and it is reached about one minute after the start of cleavage. In selecting this basis for counting time it was not forseen that the rate of the passage of the furrow would be appreciably altered in many of the fragments of the centrifuged eggs. When this phenomenon became apparent, the time of cleavage for such fragments, as recorded in Table I., was then taken as one minute after the first appearance of the cleavage furrow.

Each set of figures in the three columns, "haploid," "diploid," "difference" refers to the two halves of the same egg. In cases where one of the two fragments failed to yield a result a dash is put in its place and also in the "difference" column.

Table I.

Time to Cleavage in Minutes.

Female.	Temperature ± ¼° C.	Non-Centrifuged Controls 50% Cleaved.	Centrifuged Controls 50% Cleaved.	Non-Centrifuged Fragments.			Centrifuged Fragments.		
				Diploid.	Haploid.	Difference.	Diploid.	Haploid.	Difference.
1	20°	56.0		57.0	65.5	8.5			
		57.0		57.0	65.5	8.5			
				59.0	66.0	7.0			
				60.0	67.0	7.0			
2	21°	56.5		55.5	61.5	6.0			
				56.5	61.5	5.0			
				58.5	64.5	6.0			
				58.5	64.5	6.0			
				59.0	65.5	6.5			
3	22°	52.0					56.0	—	—
							52.0	—	—
							45.0	—	—
4	22°	52.0	52.0	46.5	52.0	5.5	49.0	56.0	7.0
				46.0	53.0	7.0	48.0	—	—
5	22°			51.0	60.0	9.0			
6	22¼°	50.0	49.5				45.0	53.0	8.0
							49.0	56.0	7.0
7	22¼°	49.0					53.0	58.0	5.0
							48.0	56.0	8.0
8	22½°	50.0		45.0	51.0	6.0			
				48.0	53.0	5.0			
				46.0	51.5	5.5			
				53.0	59.0	6.0			
				48.0	—	—			
				49.0	61.0	13.0			
				48.0	55.5	6.5			
				47.0	53.0	6.0			
				49.0	53.5	4.5			
				48.0	—	—			
				49.0	52.5	3.5			
				48.0	53.0	5.0			
				47.0	—	—			
				47.5	—	—			
				48.5	52.5	4.0			
9	22½°	50.5							
10	22½°	49.0	50.0	46.5	55.5	9.0	46.0	51.5	5.5
				48.5	54.5	6.0	—	54.0	—
							48.0	52.5	4.5

Female.	Temperature ± ¼° C.	Non-Centrifuged Controls 50% Cleaved.	Centrifuged Controls 50% Cleaved.	Non-Centrifuged Fragments.			Centrifuged Fragments.		
				Diploid.	Haploid.	Difference.	Diploid.	Haploid.	Difference.
							48.0	—	—
11	22½°	49.5		48.0	54.0	6.0	51.5	—	—
				45.0	52.0	7.0	51.5	59.0	7.5
				45.5	53.5	8.0			
				50.0	56.0	6.0			
				48.0	55.0	7.0			
				55.0	60.5	5.5			
12	22¾°	48.0	47.0				45.0	51.0	6.0
							48.5	—	—
							45.0	52.0	7.0
							44.5	53.0	8.5
							—	51.0	—
							47.5	—	—
							46.0	54.0	8.0
13	23°	49.0 49.0							
14	23°	43.5	43.5				—	51.0	—
							46.0	55.0	9.0
15	23°	46.0	47.0				43.0	51.0	8.0
16	23½°	44.5	45.5	42.0	48.5	6.5	46.0	52.0	6.0
		44.5		42.0	—	—	42.0	49.0	7.0
				43.0	50.0	7.0	44.0	50.0	6.0
							47.0	50.0	3.0
17	23½°	46.0	48.0	46.0	52.0	6.0	42.0	49.0	7.0
				45.0	50.0	5.0	52.0	57.0	5.0
							44.0	51.0	7.0
							43.0	50.0	7.0
18	23½°	46.0	46.0				49.5	—	—
							55.5	63.5	8.0
							45.5	52.0	6.5
							48.5	56.5	8.0
19	24°	44.0	46.0	43.0	53.0	10.0			
				49.5	55.5	6.0			
				44.0	48.5	4.5			
20	24½°	43.0 41.0							
					Average	6.5		Average	6.8

Effect of the Stratified Materials.

It is observed in Table I. that the averages of the difference in time from fertilization to cleavage of the diploid and the haploid fragments in the two cases, non-centrifuged and centrifuged, are practically the same: 6.5 minutes (38 cases) for the non-centrifuged, and 6.8 minutes (25 cases) for the centrifuged. While there are not enough cases to establish a negligible probable error, it is evident that the effect of the granules on the cleavage rate is either very slight or entirely absent.

In averaging these differences in cleavage rate the differences in temperature of the different experiments have been ignored, because in all cases the two fragments compared were from the same egg and at the same temperature. Therefore while the actual time lapse to cleavage varies considerably within the 4½° temperature range, as seen in Table I., both fragments are speeded up or slowed down so that the difference is probably not changed within the limits with which we are at present concerned, considering that the averages compared are derived from fairly comparable distributions over the temperature range.

The difference between the cleavage rate of haploid and diploid fragments is practically the same in centrifuged and non-centrifuged eggs. But it is still possible that the two fragments of the centrifuged egg, for example, are both delayed almost exactly the same amount compared with those of the non-centrifuged. In this case the differences between haploid and diploid fragments in the two cases would still be the same. The diploid centrifuged fragment might conceivably be delayed by a shortage of granules and the haploid centrifuged fragment by increased viscosity. If this were the case, the results would indicate such an exact compensation from independent causes that such an equal delay does not seem likely. The direct comparison must be made with cases in which the temperature is the same throughout within close limits. This condition is realized in eggs from a particular female. The cases which best fulfil the requirements are those from females 4, 10, 11, 16, and 17 (Table I.). In each of these five cases the averages are taken of each the haploid and the diploid centrifuged and the haploid and the diploid non-centrifuged fragments. The five averages of the haploid centrifuged fragments from the five females are then averaged, thus giving equal weight to each temperature. In the same way the other three sets (diploid centrifuged, haploid non-centrifuged, and diploid non-centrifuged), are also averaged. In this way error from unequal distribution over the temperature range is avoided. The results are: average time to cleavage of haploid fragments from non-centrifuged eggs, 52.6 minutes (based on 14 cases); haploid fragments from centrifuged eggs, 53.9 minutes (13 cases); diploid fragments from non-centrifuged eggs, 46.0 minutes (15 cases); diploid fragments from centrifuged eggs, 47.4 minutes (15 cases). This shows a slight delay on the part of both fragments of the centrifuged egg but not much greater than is found for whole centrifuged eggs (see below) and not as great as the limits of error for so few cases. Here we are comparing fragments from different eggs. A sample of eggs has a probability distribution of time lapse to cleavage covering several minutes. Hence from this we can only conclude that any delay of both fragments of the centrifuged egg is at least very slight.

From the ten females numbered 4, 6, 10, 12, 14, 15, 16, 17, 18, 19, centrifuged and non-centrifuged controls, both at the same temperature, were timed for 50 per cent. cleavage. The average time is 46.9 minutes for non-centrifuged, 47.4 minutes for centrifuged eggs.

Effect of Amount of Nuclear Material

It has been shown that diploid fragments cleave sooner than haploid fragments. This seems to indicate that the ratio between nuclear and cytoplasmic amounts is a determining factor in cleavage rate. But since the sperm contributes a precursor to the amphiaster as well as a nucleus it may be that the derivatives of the sperm are not exactly equivalent to the egg nucleus in effect on cleavage rate. A comparison between the cleavage rates of normal uncut diploid controls and diploid fragments bears on this point. Here the nuclear content is identical, consisting of an egg and a sperm nucleus, but the amount of cytoplasm is half in the case of the fragment. For this comparison it is necessary to have approximately constant temperature, so two large groups of cases will be selected within which the temperature range is small. The first group includes results from females 3 to 11 inclusive. The temperature range is 22° C. to 22½° C. The second group includes females 12 to 18 inclusive, with temperature range from 22¾° C. to 23½° C. The results are shown in Table II. It is seen that diploid fragments cleave sooner than normal eggs in spite of the injury of cutting.

The viscosity of the two fragments "clear" and "granular" is found to differ markedly, but no quantitative measure of this difference has been made. The evidence for difference in viscosity.

and the results and implications are considered in the latter part of the last section (Discussion).

TABLE II.

Cases.	Group I 22°–$22\frac{1}{2}^\circ$	Average time (minutes)
11	whole egg controls (50% cleaved)	50.3
40	diploid fragments	48.3
31	haploid fragments	54.8
	Group II $22\frac{3}{4}^\circ$–$23\frac{1}{2}^\circ$	
15	whole egg controls (50% cleaved)	46.2
25	diploid fragments	45.7
23	haploid fragments	52.1

Development.

Lyon (1906) found that stratified *Arbacia* eggs develop normally, and Morgan and Spooner (1909) found that they do so in accordance with the original polarity of the egg and without regard to the planes of stratification of the egg or the concentrated zones of materials which are moved by the centrifuge.

In the case of uncut eggs it can be readily observed that a large amount of concentrated material is redistributed in most of the eggs within three quarters of an hour. If they are fertilized the redistribution before cleavage is even more pronounced, aided no doubt by the protoplasmic flows which precede cleavage. It is entirely possible that fragments from eggs cut immediately after centrifuging would show developmental alterations not present in uncut eggs. But time was not available during the present work to follow the development of these fragments. Several early blastulae were incidentally observed from each kind of fragment but they were not followed further. However fragments from centrifuged eggs of the Pacific coast starfish *Patiria miniata* have been followed. These eggs cannot be stratified as sharply as Arbacia eggs, but after 35 minutes at 4,000 r.p.m. with 20 cm. radius (3,600 gravities) the eggs of most females become quite clear at one end. Complete gastrulae have been reared from clear fragments as well as from their mates. In this case there is a complication, which will not be dealt with here, concerning localized formative stuff (Whitaker, 1928). There is also an unusual nuclear phenomenon in this egg. The nuclei from the polar bodies move back into the egg when it is centrifuged and form a polyploid egg which divides to 6 or 8 cells a little before the normal egg divides to 2 cells. The behavior of these nuclei will be considered at another time.

Discussion.

Cleavage and Oxidation.

Warburg (1913) found that when unfertilized sea urchin eggs are ground in a mortar with sand, oxygen consumption continues independently of the living condition for some hours at about the same rate as in normal unfertilized eggs. He found further (1914) that if eggs with membranes broken by shaking are centrifuged so that the granules are concentrated, about 82 per cent. of the oxidation takes place in the sediment containing the granules.

If the granules in the living egg act as catalysts of oxidation as in the crushed eggs, it seemed possible that a difference in developmental rate might be found in the two fragments, granular and non-granular. On the other hand it has been known for some time that developmental rate[2] and oxidation rate do not proceed very closely together. Crozier (1925) points out that the two processes are essentially dissimilar since development must depend on synthetic processes. It is not then to be expected that oxidation rate is normally the limiting factor in cleavage rate. But it may become so experimentally, as shown for example by Loeb (1896) when he suppressed cleavage in sea urchin eggs by complete absence of oxygen. Therefore, if the granules are catalysts of oxidations, if their concentration were sufficiently diminished the oxidations which they catalyze might become limiting to cleavage rate. Such an effect was looked for especially in the " clear " fragments of completely stratified eggs. But such limitation was not found. Even when excluded to a very great extent the granular material of the egg does not become limiting to cleavage rate;

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[2] Distinction should be made between cleavage rate and growth rate in the sense of increase in weight. In the early cleavage stages weight is in all probability lost, except for the intake of water since CO_2 and heat are produced and no food is taken in. However synthetic processes are taking place since embryological differentiation is going on.

nor does it appreciably alter cleavage rate when its concentration is approximately doubled.

Amberson (1928) has recently published results on the effect of reduced oxygen pressure on cleavage rate in *Arbacia* eggs. He found that the oxygen pressure must be reduced to 20 mm. Hg. before the rate of oxygen consumption in fertilized *Arbacia* eggs begins to decline sharply. But not until the oxygen pressure is further reduced to 11 mm. Hg. is the cleavage rate retarded. At this pressure the oxygen consumption is reduced to about one half normal. Cleavage is suppressed completely at about 4 mm. Hg. These results prove directly that oxygen consumption must be reduced to about one half before it becomes limiting to the reactions which lead to cleavage.

If as much as 80 per cent. of the oxidation takes place in the granular half of the egg, it must be reduced to 20 per cent. in the "clear" fragment. This 20 per cent. is less than half normal (*i.e.* half of 50 per cent.). From Amberson's results, which were not published until after my own work was done, it can be estimated that the "clear" fragment ought to show retardation of cleavage rate, if oxygen consumption is reduced to less than half by the removal of the granules.

Viscosity.

The two halves of the cut centrifuged egg differ in viscosity as well as in content of formed bodies. The pigmented half is considerably more viscous as shown in several ways. After the egg is cut, the "clear" fragments round up more quickly than the "granular." When fragments are ruptured, long extensions flow out from "clear" fragments while smooth bulges result on "granular" fragments. A good rough comparative measure, in spite of the danger of subjective interpretation, can be made by prodding with the needle and observing the rate of flow in response to distortion. It can be concluded with reliability that there is a gradient of viscosity perpendicular to the strata with a maximum in the pigment cap.

It has been shown that the amount of granular material contained in the fragment has practically no effect on the time lapse until cleavage starts. But there is a marked difference between "clear" and "granular" fragments in the time required for the act of cleavage to be completed. The "clear" liquid fragment cleaves completely in less than a minute, often in less than half a minute. It does so more quickly than the normal egg. The pigmented viscous fragment takes from one or two to six minutes or sometimes more to cleave. The furrow usually starts on the least viscous side of the pigmented fragment and follows slowly along the gradient of viscosity, *i.e.*, passes through the pigmented cap last. This suggests a connection between the viscosity and the rate of passage of the cleavage furrow (perhaps by an effect on the mechanics of cleavage). But it is significant that the rate of the steps leading to the start of cleavage is not appreciably affected. The precleavage processes are neither retarded nor accelerated by change of viscosity within the limits of change brought about in these experiments. The exact nature of the gradient of viscosity is not known. There is no evidence to show how much it is due to a mere mechanical displacement of protoplasm by more solid particles or to what extent it is due to transference of materials taking part in a more truly viscous gel structure.

SUMMARY AND CONCLUSION.

1. Fragments of centrifuged *Arbacia* eggs, "clear" and "granlar," start to cleave at practically the same time after fertilization as fragments of non-centrifuged eggs.

2. The cleavage furrow passes more slowly through the "granular" fragments than through the "clear" fragments. It passes through the fragments of non-centrifuged eggs at an intermediate rate. The "granular" fragment is considerably more viscous than the "clear" fragment.

3. The ratio of the amounts of nuclear material and cytoplasm is a determining factor in cleavage rate. Half an egg containing both the egg nucleus and a sperm nucleus cleaves sooner than the normal diploid egg in spite of the injury from cutting. The normal egg cleaves sooner than half an egg containing only the sperm nucleus.

BIBLIOGRAPHY.

Amberson, W. R.

'28 The Influence of Oxygen Tension upon the Respiration of Unicellular Organisms. Biol. Bull., Vol. LV., no. 2.

Crozier, W. J.

'25 On Biological Oxidations as Function of Temperature. Jour. Gen. Physiol., Vol. VII., p. 189.

Loeb, J.

'96 Untersuchungen über die physiologischen Wirkungen des Sauerstoffmangels. Pflügers Arch., Bd. LXII., s. 249.

Lyon, E. P.

'06 Some Results of Centrifugalizing the Egg of *Arbacia*. Amer. Jour. Physiol., Vol. XV.

Morgan, T. H. and Spooner, G. B.

'09 The Polarity of the Centrifuged Egg. Arch. Entw.-mech., Vol. XXIV.

Tennent, D. H., Taylor, C. V., and Whitaker, D. M.

'29 An Investigation on Organization in a Sea-urchin Egg. Carnegie Inst. of Wash. Pub., No. 391, p. 1.

Warburg, O.

'13 Ueber sauerstoffatmende Körnchen aus Leberzellen und über Sauerstoffatmung in Berkefeld-Filtraten wässriger Leberextrakte. Pflügers Arch. Ges. Physiol., bd. 154, s. 599.

Warburg, O.

'14 Zellstruktur und Oxydationsgeschwindigkeit nach Versuchen am Seeigelei. Pflügers Arch. Ges. Physiol., bd. 158, seite 189.

Whitaker, D. M.

'28 Localization in the Starfish Egg and Fusion of Blastulae from Egg Fragments. Physiol. Zoöl., Vol. 1, no. 1, p. 55.

COLOR INHERITANCE IN LARVÆ OF *CULEX PIPIENS* LINN.

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Introduction.

In collecting mosquito larvæ from the various types of breeding places, one is often struck by the great variations in color which occur. Many who have collected mosquito larvæ—either Anopheline or Culicine—may recall that there is often a striking similarity between the color of the fully-grown larva and the color of its immediate environment. Larvæ found in water containing large quantities of green organisms will often be brilliant green themselves, while larvæ of the same species if observed in muddy water will be brown. This is not a strict parallelism, however, for one may sometimes find larvæ whose color stands out in contrast to the color of their environment. Two interpretations may be offered of the fact that there is often observed a similarity in the color of the larvæ and the color of their environment. One is the suggestion that the larva ingests the organisms of the water and that the predominating color of the latter manifests itself through the rather transparent integument of the insect. The other is that colors are actually inherited and that the correlation mentioned above is due to the rapid action of natural selection in favor of those larvæ which resemble most closely their environment. Either of these hypotheses is difficult to test unless one employs a species of mosquito which can be bred in captivity from generation to generation. As shown in my previous publications (1927, 1929*a*, 1929*b*) *Culex pipiens* is a very good species for laboratory experimentation. I have now bred this species through 15 generations and in that time have seen it adapt itself to its laboratory environment in various ways. First, I have shown (1929*b*) that the species must become adapted, through selection, to copulating in captivity, since it copulates in nature while swarming. My strain of mosquitoes will now breed in a space no larger than a test tube, and practically all of the breeding is done in cages constructed from lantern globes. I have also been able to shorten the life-cycle to less than half of what it was when originally brought in from the field. Under optimum conditions of food and temperature larvæ will become fully grown in five days from the time the eggs are laid. While the species thrives better when the females can secure blood meals, I have shown (1929*a*) that the species may be kept breeding on a purely vegetable diet, and recently I have observed one instance of the laying of viable ova by a mosquito in less than 24 hours after her emergence from the pupa and having taken no food of any kind (1929*a,* footnote).

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Therefore, when I first observed some vividly green larvæ in my stock I decided to try to determine which of the two hypotheses about larval color would better fit the facts.

INFLUENCE OF ENVIRONMENTAL FACTORS.

After I had inbred this species for three generations (for purposes shown in another paper (1929*b*)), I noticed the sudden appearance of green larvæ in large numbers. In fact, every larva in one particular breeding bowl was found to be green on the day before pupation. The color persisted in the pupa, although it became obscured as the pigment of the adult tissue developed. Upon emergence the green color could still be observed in the abdomen of the adult. Since I had on hand a bowl of larvæ in which I had seen no trace of green—all of them being reddish brown in color—I decided to save the progenies of the two lots. When ova from each strain were obtained they were allowed to hatch and the larvæ were placed on opposite sides of a bolting cloth partition within a breeding bowl. Stock food consisting of two parts of milk powder to one part of dried blood serum was added in small quantities each day. Thus the larvæ were under practically identical conditions of temperature, food, hydrogen ion concentration, viscosity, etc. In due time the larvæ became fully grown. All of those on one side of the partition which were from brown stock were brown in color and showed no hint of green coloration. All of those on the other side were vividly green as had been their parents. This seemed to be conclusive proof that environmental factors did not determine the color of the larvæ directly, at least.

Hereditary Behavior.

After the two stocks—brown and green—had been bred three generations and were still breeding true, a male from the brown stock was mated with a female from the green stock. The fully grown larvæ from this cross were "normal" in color; that is, they were predominantly brown with a tint of green visible on the clear parts of the abdomen. The adults from these larvæ were inbred and 10 lots of ova obtained. When they were ready to pupate they were separated into two lots—one containing only those of a pure green color, the other, those showing a pure brown or a color predominantly brown. The results of this cross were as follows:

	Number	Percentage	Ratio
Green	149	25.38	1 : 2.94
Brown	438	74.62	

The brown lot was then subdivided into two groups one of which contained larvæ without any trace of green and the other with traces of green. Due to the rapid pupation of the larvæ it was impossible to determine in this experiment the percentages in the two groups. However, the ones suspected of being heterozygous for these colors were inbred and it was found that the larvæ in this generation were composed of pure green, pure brown, and "normal" individuals. These numbers were too small to be significant quantitatively and are not recorded.

Nature of the Color.

Aside from the experiments described above which show that microorganisms did not determine the color of the larvæ, a number of observations made from larval dissections showed directly that the color was due to refraction of light from the globules in the fat body. There were no microorganisms observed in the regions showing the colors in question. The fat body, however, is made up of very numerous globules resembling oil which in the aggregate give the colors characteristic of the two strains. The distribution of these colors follows closely the distribution of the fat body within the larva. I am of the opinion that the differences in color of these 4th stage larvæ were due to hereditary differences in the physical or chemical nature of the fat body. One should not confuse the colors about which I have written, with colors in the integument due to pigments.

CONCLUSIONS.

On the basis of the results of experiments upon regulation of the environmental factors, upon cross breeding, and upon larval dissections it is concluded that the brown and green colors observed in 4th stage larvæ of *Culex pipiens* are due to hereditary differences rather than to the direct influence of the environment. It is believed that these differences are concerned with the properties of the fat body of the insect—probably with its index of refraction.

LITERATURE.

Huff, C. G.

'27 Studies on the Infectivity of Plasmodia of Birds for Mosquitoes, with Special Reference to the Problem of Immunity in the Mosquito. Am. Jour. Hygiene, 7 (6): 706–734.

'29*a* Ovulation Requirements of *Culex pipiens* Linn. BIOL. BULL., 56 (5): 347–350.

'29*b* The effects of Selection upon Susceptibility to Bird Malaria in *Culex pipiens.* Ann. of Trop. Med. and Parasit.

GROWTH REGULATING SUBSTANCES IN ECHINODERM LARVÆ.

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In general we apply the term growth to the progressive development of an organism from the earliest stages to a period approaching maturity. From this series of changes I have selected, as representative, two brief periods namely the early cleavages, and the period of rapid growth in length of the arms of the pluteus during the second and third day of their development. The experiments fall into three groups, the first of which has to do with the direct contact effects of developing eggs upon one another, and the character of the influence of their secretions upon these two periods of growth; the second group deals with the effects of extracts of eggs and of larvæ upon the development of other eggs, and the third is concerned with the possible removal by adsorption and the subsequent recovery of any growth-accelerating and growth-inhibiting substances that may be secreted.

These investigations were begun at Woods Hole in July, 1926, and were continued in the summer of 1927 at Naples where I also undertook a series of experiments with adsorption, the results of which indicate that it is possible by this means to eliminate some of the factors which are involved in the inhibition of developmental processes. It is with pleasure that I acknowledge my indebtedness to Professor Frank R. Lillie who was at that time director of the Marine Biological Laboratory at Wood Hole. Also I am glad of this opportunity to thank the Association for the Promotion of Scientific Research among Women, for the use of the American Woman's Table in the station at Naples. To the director Dr. R. Dohrn I am once more grateful for the many courtesies he has always extended to me while I was a guest of the station.

Materials and Methods.

The forms used at Woods Hole were *Arbacia* and *Asterias,* both being abundant there during the summer months. At Naples the experiments were made on the eggs of *Strongylocentrotus, Arbacia,* and *Sphærechinus.* So far as possible eggs and sperms for each series were taken from the same pair of individuals. At Woods Hole the urchins were opened and allowed to shed their eggs and sperm into shallow dishes, with the Mediterranean species the reproductive organs were removed, and the eggs were shaken immediately into fresh sea water. Sperm suspensions were made in the usual manner and the minimal amount used for insemination. At Woods Hole the developing eggs were kept in finger bowls, on a shelf in the laboratory, but in Naples, owing to the great heat it was found that they developed more normally when the dishes which contained them were surrounded with running water. Further methods will be described in connection with individual experiments.

I. The Contact Effect of Developing Eggs and of Larvæ upon the Rate of Cleavage, and upon Growth in Length of Arms of Plutei.

(*a*) *The Influence of Direct Contact.*—It is a matter of ordinary observation that growth of lower organisms, both plant and animal, takes place more rapidly in a crowded medium than it does in a thinly populated culture, provided the food supply is not exhausted. The acceleration in many forms is directly related to the presence of bacteria, and is maintained as long as they are present in large quantities. It was first demonstrated by Wildiers ('01) that isolated yeast cell do not grow and divide, but when a number of cells are added the development proceeds in the normal manner. He pointed out that a secretion of the yeast cells which he termed *bios,* will also produce the same effect. Burrows ('25) and others who have worked extensively with tissue cultures, have observed that isolated cells of the body grow less well *in vitro* than when crowded together. In the fertilized egg of the sea urchin there is sufficient food material to last until the free swimming larvæ develop. There is then, in these cells no need to draw nutritive materials from the sea water, and food is probably not taken in until the digestive tract is formed. There is strong evidence, however, that the egg secretes substances not necessarily nutritive in nature which greatly modify the composition of the sea water. Whether these substances contain growth hormones, vitamines, or catalysts, it is the purpose of this study to determine.

In order to test the effect of direct contact of one egg upon another the following experiment was made. As soon as the fertilization membrane appeared the inseminated eggs were washed several times in fresh sea water to remove the fertilizin and any sperm remaining in the water. Single eggs were placed in ten to fifty cc. of sea water, in another series five eggs were put in a corresponding volume of water, in another ten eggs, and so on. The rate of cleavage in these eggs was compared with that of several hundred eggs in an equal amount of water. The rate in the isolated eggs was so nearly that of those crowded together that the coefficient of variation in one series was about the same as that of another. All of the eggs reached the free swimming stage at the same time, but the percentage of abnormal eggs was greater when large numbers developed in a proportionally small amount of water. This was no doubt largely due to the excretion of carbon dioxide. Mortality was increased also under these conditions, many of the eggs failing to reach the blastula and gastrula stages.

If, as Robertson ('23) suggests, a catalyst is freed at each nuclear division it would be probable that at the end of the third cleavage only a very minute quantity would have accumulated, but at the end of the 256-cell stage a considerable amount might have passed out into the surrounding medium. In order that such a diffused substance might reach younger developing eggs without direct contact with the older ones, and also to avoid any possibility of confusing the two sets of eggs they were separated by parchment thimbles, collodion membranes, or extremely fine bolting cloth. At first it seemed advisable to bubble air through the water in which the eggs were developing, but this method was abandoned as soon as it was discovered that the eggs in the vessels through which air passed showed a much lower percentage of normal development than those without air. In Table I. the results of a series of these experiments are grouped.

Table I.

Type of Membrane Employed.	Stage in Older Eggs.	Min. after Fert. to Cleavage.			Control.		
		I	II	III	I	II	III
Parchment thimble..........	8-cell	50	70	90	55	80	95
	"	50	75	85	50	80	105
Collodion membrane........	Gastrulæ	45	60	90	45	60	90
	"	50	65	85	45	60	90
Bolting Cloth..............	Plutei	59	94	108	60	95	110
	"	60	95	120	61	93	120

The rate of cleavage in eggs separated from older stages by means of a membrane. The average length of time in minutes was reckoned from insemination until fifty per cent. were divided.

For these experiments the eggs were washed immediately after membrane formation, and placed in the parchment thimble, or in a glass tube 4 cm. in diameter, the lower end of which was covered with the membrane used for the particular series. These dialyzers were then submerged in dishes in which older eggs or embryos had developed up to a given time. As the table shows, there was very little variation in the time of the appearance of the cleavage furrows when the eggs in the dialyzers were compared with the control, but there is some indication of acceleration in the rate of cleavage in the younger eggs, and this acceleration is greater when the membranes were used than when the two sets of eggs were separated by bolting cloth. Whether or not the accelerating substance given off by the older eggs passes through the membrane while the inhibiting substances are held back, remains to be determined when more is known of their nature.

(*b*) *The Effect of Embryo-water upon the Rate of Cleavage and of Growth in Length of Plutei.*—In still another set of experiments eggs were allowed to segment in water in which other eggs had developed up to the gastrula and plutei stages respectively. Springer ('22) used this method in an attempt to determine if so-called formative substances pass from eggs or from larvæ into the surrounding water. She observed no acceleration in rate of cleavage, and her results do not prove or disprove the existence of growth-promoting substances: as she suggests, such substances if present in the egg may not pass into solution in sea water, or if they do they may be too complex in character to be disassociated from the molecules that hold them. Even if they do pass into the water the cells of the eggs developing in their presence may not be permeable to them. In Fig. 1 the rate of the appearance of the first three furrows is shown in contrast to the rate of cleavage of eggs in embryo-water. The delay here would indicate that inhibiting substances had accumulated in the water.

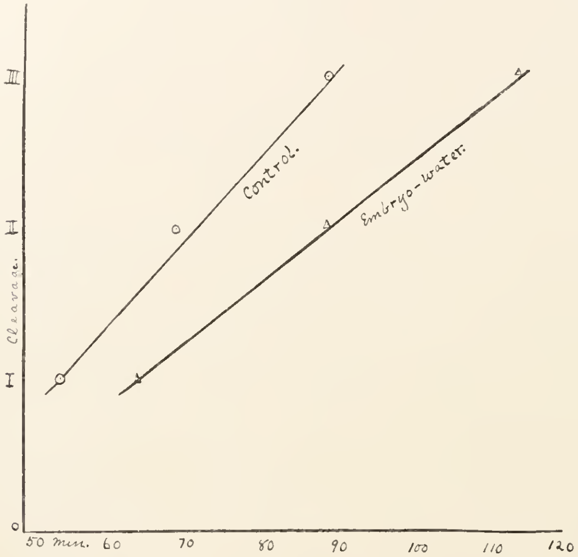

Fig. 1. Comparison of the rate of appearance of the first three cleavage planes (I., II., and III.) in normal eggs (⊙), and in eggs allowed to develop in Embryo-water (△). The abscissa shows the number of minutes from insemination to the appearance of the furrow in 50 per cent. of the eggs. The ordinate gives the cleavages.

Vernon ('95) found that larvæ grown in water in which other larvæ had previously developed were seven per cent. smaller than normal, showing that some of the products of metabolism exert a deleterious effect on growth, but he found that urea and uric acid caused increase in size. He states that a small excess of carbon dioxide stimulated growth, but Haywood ('27) has found that excess of carbon dioxide delays the appearance of the first furrow. In Fig. 2 the average rate of growth in length of the plutei, measuring from the base of the body to the tip of one of the anal arms is given. The most rapid growth in length takes place during the second and the third days. It will be observed that the greatest delay in growth appears when the eggs develop to the plutei stage in plutei-water, and the delay is less when the older plutei are present, but when younger and older eggs develop together both the young and the old larvæ show retarded growth. Boiling the plutei-water removes the inhibiting effect, and such water standing for a week after the larvæ have been removed seems to have the same effect. We conclude then that embryo-water contains substances that inhibit growth. But some of the inhibiting power is counteracted when living larvæ are present, and if the water in which larvæ have developed is boiled or is allowed to stand long enough, after the larvæ have been removed, the inhibiting effect

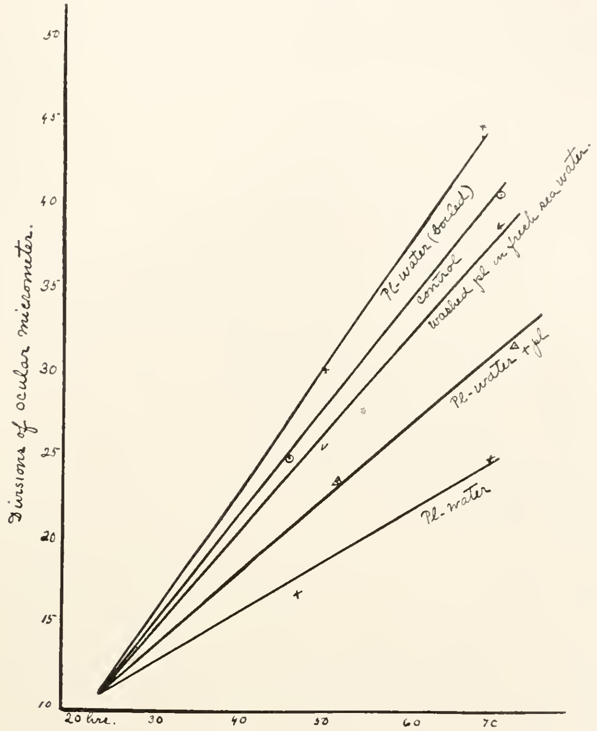

FIG. 2. Comparison of the rate of growth in length of plutei, the abscissa shows the number of hours after insemination, the ordinate, the length of the plutei (body plus anal arm), in divisions of the ocular micrometer.

is lost. Fig. 2 shows a slight acceleration in the rate of growth in the last two cases. The apparent acceleration in the case of the boiled plutei-water may be due to the adding of distilled water to make up for the loss in boiling.

II. The Effect of Extracts of the Egg and of Larvæ upon the Rate of Cleavage, and upon Later Growth.

As Springer has shown, extracts of eggs and of larvæ made by grinding them with sand and washing out with sea water always inhibit the rate of development. So far I have been unable by dialyzing to remove any substance which stimulates growth, but the results from the following experiments seem to indicate that such substances exist, and they may be masked by the stronger and more abundant growth-inhibiting factors.

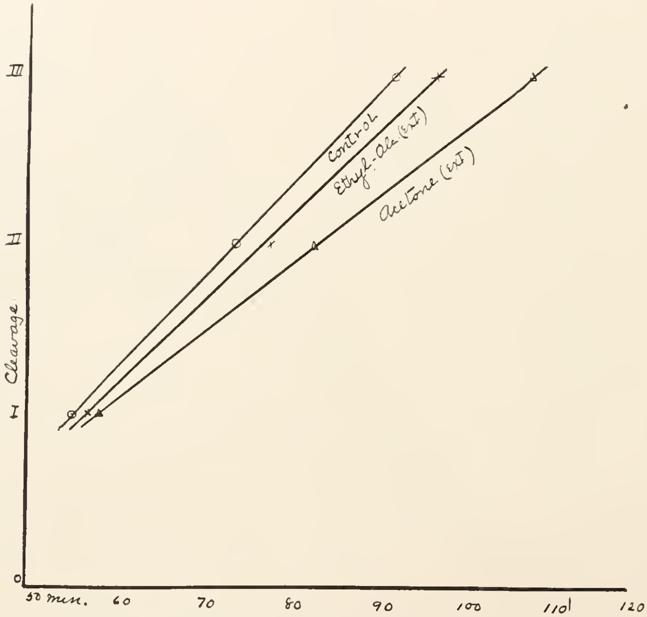

FIG. 3. Time of appearance of the first three furrows in eggs which have developed in alcoholic extract (×), in acetone extract (△), and in sea water (⊙).

In Fig. 3 the cleavage rate is shown for eggs in 90 per cent. ethyl alcoholic and 50 per cent. acetone extract of plutei. It will be seen at once that the rate is slowed in the alcohol, and slower still in the acetone. In these extract the plutei were placed in the alcohol or acetone, then the mixture without grinding the plutei was evaporated to dryness, and to a given quantity sea water was added. In Fig. 4 the rate of growth in length is given for the second and third day. Here again it is evident that there is greater delay in the acetone extract than there is in the alcohol. If however, the fats are removed from the acetone extract, and a water solution made from the residue there is evidence that the inhibiting effect is removed. This may be seen in Fig. 5.

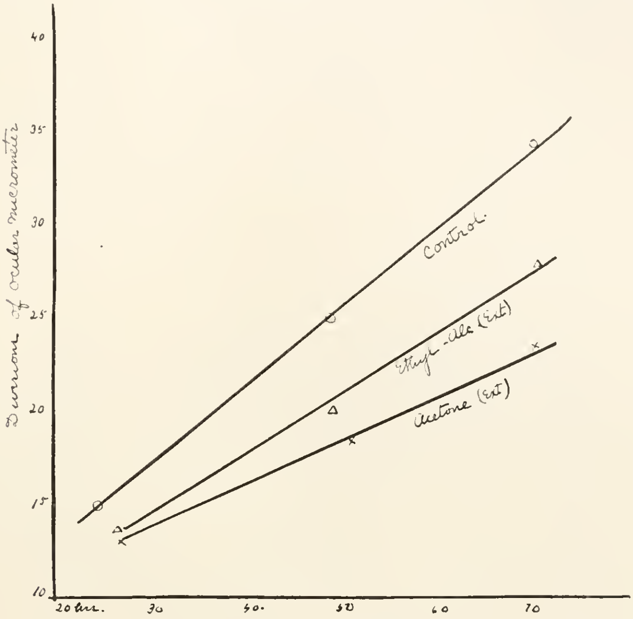

FIG. 4. Growth in length of plutei in the same extracts used in Fig. 3.

Heaton ('26) found that yeast and liver cells contain both a substance that inhibits growth and one that promotes it. In extracting these substances he discovered that the factor promoting growth of epithelial cells could be extracted with 97 per cent. alcohol, while the growth-inhibiting substance was soluble in alcohol only up to 75 per cent. I have used these two concentrations of alcohol in making extracts of the plutei of *Strongylocentrotus* and *Arbacia,* but so far I have not found that the extracts differ in their effect other than that the solutions from the stronger alcohol had a more marked effect than those from the weaker.

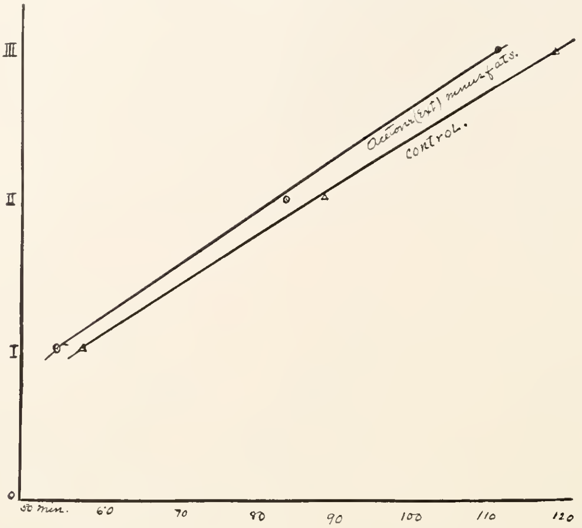

Fig. 5. Cleavage rate in eggs treated with acetone extract from which the fat has been partially removed.

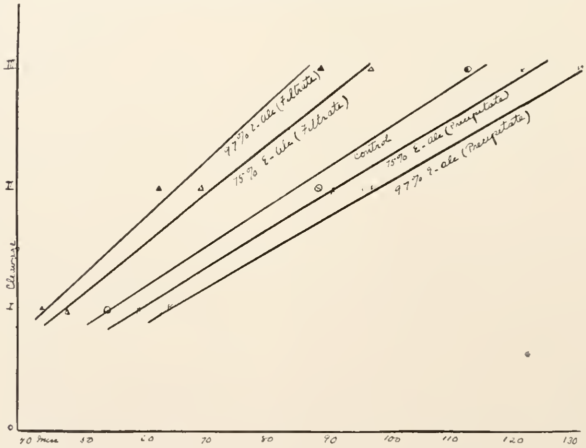

Fig. 6. A comparison of the effect of the filtered alcoholic extracts of plutei (▲ and △) with that of the precipitate (X and V).

After allowing the alcohol to evaporate to dryness on the plutei, sea water was added and the precipitate together with the skeletons and the debris removed by filtering. Fig. 6 demonstrates very clearly that the filtrate and the precipitate differ considerably in their influence on the rate of cleavage, the precipitate delaying it and the filtrate accelerating it.

III. The Removal of Inhibiting Substances by Adsorption.

In order to remove substances secreted by eggs at the time of fertilization, Glaser ('21) used charcoal, and found that the eggs were completely sterilized in three to four hours. Hinrichs ('27) also employed charcoal to adsorb fertilizin, which she later recovered with HCl. Baker and Carrel ('27) made use of charcoal,

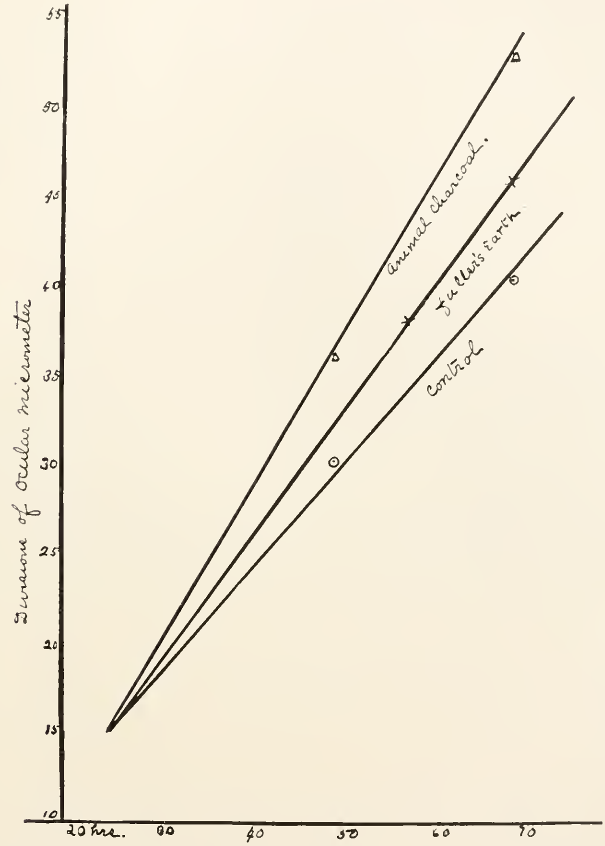

FIG. 7. Growth in length of plutei in the presence of animal charcoal (△), and fuller's earth (×), compared with those growing in sea water (⊙).

kaolin, and other adsorbants to remove protein substances from embryo-tissue extract, but they have not yet been successful in recovering the active substances again. In Fig. 7 the rate of growth of plutei is given when animal charcoal and fuller's earth were used as adsorbents. There is evidence here that both of these substances remove some inhibiting influence from the sea water in which the eggs are developing. There is also less abnormality, and the mortality is decreased. The pH of the water was ascertained in these experiments and it varied so slightly from that of the control that it did not seem to be a determining factor. Up to the present time I have not succeeded in removing from the adsorbents any substance which affects the rate of growth of eggs subjected to its influence. It is hoped through longer and more exact investigations to isolate both the growth-inhibiting and the growth-promoting substances in such a form that they may be employed to regulate the development of other eggs.

Summary and Conclusions.

1. The eggs and larvæ of the sea urchin and starfish contain growth regulating substances. These substances pass out into the surrounding water during segmentation, and the later stages of larval development.

2. The nature of these substances has not been determined, but there is some experimental evidence in favor of the conclusion that the inhibiting substances are associated with the lipoid constituents, and the accelerating factor is contained in the protein molecule.

3. After the removal of the fats from the extracts of gastrulæ and plutei, a solution of the residue in sea water exerts a slightly stimulating effect on growth; when the alcoholic extracts are filtered, and the precipitate removed the filtrate has the same accelerating effect. Acetone extracts of eggs and of larvæ, as well as alcoholic extracts, if used in pure form, are inhibitive in their influence.

4. The retarding effect of secretions of growing embryos is removed in the presence of animal charcoal and fuller's earth. The percentage of normal larvæ resulting from eggs grown in the presence of these adsorbents is greatly increased while mortality is decreased.

BIBLIOGRAPHY.

Vernon, H. M.

'95 The Effect of Environment on the Development of Echinoderm Larvæ. Phil. Trans. Roy. Soc. London, Vol. 36, p. 577.

Wildiers, E.

'01 Nouvelle Substance indispensible au Development de la Levure. La Cellule, Vol. 18, p. 311.

Glaser, O.

'21 The Duality of Egg-Secretion. Amer. Nat., Vol. 55, pp. 368–373.

Springer, M. G.

'22 The Effect upon Developing Eggs of Extracts of the Same Species. Biol. Bull., Vol. 43, pp. 75–97.

Robertson, T. B.

'23 The Chemical Basis of Growth and Senescence. Philadelphia and London.

Burrows, M. T.

'25 The Action of Oils in the Production of Tumors. Archives of Internal Medicine, Vol. 36, pp. 293–322.

Heaton, T. B.

'26 The Nutritive Requirements of Growing Cells. Jour. Path. and Bact., Vol. 29, pp. 293–307.

Baker, L. E., and Carrel, A.

'27 The Chemical Nature of Substances required for Cell Multiplication. Studies from the Rock. Inst. for Med. Res., Vol. 59, p. 501.

Haywood, C.

'27 Carbon Dioxide as a Narcotic Agent. I. Biol. Bull., Vol. 53, pp. 450–465.

Hinrichs, M. A.

'27 Ultraviolet Radiation and the Fertilization Reaction in Arbacia punctulata. Biol. Bull., Vol. 58, pp. 416–437.

STUDIES ON TRANSPLANTATION IN *PLANARIA*.

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During the last two years, the writer has been engaged in a study of transplantation in *Planaria*. The present paper is a brief statement of some of the more important results obtained thus far.

The work was undertaken in the hope of throwing light on various questions. For example: may a grafted piece act as an "organizer" or "reorganizer"? Is its effect species-specific? On what factors does the fate of the graft depend?

Materials and Methods.

Planaria dorotocephala Woodworth, and *Planaria maculata* Leidy were the animals used in the work herein reported. The size of the worms used ranges from ten to twenty-two millimeters in length. A weak aqueous solution of chloretone (ca. M/100) was used, in most cases as anaesthetic. The donors were not anaesthetized. In some cases neither the host nor the donor was anaesthetized. In some of the experiments, a triangular hole was made through the body of the host by means of a sharp scalpel at the desired level of the body and a triangular piece of about the same size as the hole from a certain level of the body of the donor was inserted into the hole by means of a capillary pipet, while in other series of experiments a circular hole was made by means of a sharp capillary pipet. The advantage of using triangular pieces and triangular holes in this experiment is very obvious, in as much as the polarity of the graft and of the host was one of the points under investigation. The operated worms were kept in the dark in moist chambers consisting of fingerbowls and small watch glasses with very little well-water, only enough to keep the worms moist, for about twelve to twenty-four hours immediately following the operation. After this length of time, those with successful grafts were examined under a binocular microscope; counted, recorded and transferred into fingerbowls with ca. 200 cc. fresh well-water, covered with glass plates and kept in the dark in the usual way. In some of the experiments, the host was beheaded to keep it relatively quiet and thus increase the per cent. of "takes," while in other cases, the host was not beheaded. Both homoioplastic transplantation and heteroplastic transplantation were performed the latter between *Planaria dorotocephala* and *Planaria maculata*. In some of the experiments *P. maculata* was the host, while in others the host was *Planaria dorotocephala*. Head, prepharyngeal, pharyngeal, postpharyngeal, and tail regions were the different levels of the body that have been used both as graft and as region of insertion in these experiments. The grafted piece was either transplanted without its axis being rotated, or the axis of the transplanted piece was rotated 60–180 degrees, and in some cases, the piece was grafted with its dorsal surface toward the ventral surface of the host. Histological studies of the results are being made and will be reported elsewhere.

Experiments and Results.

Portions of the head (Figs. 1, 2, 3) of *Planaria dorotocephala* similar to those used as head grafts were isolated. Result: As a rule, these isolated portions of the head, if not too small, remained alive for several days or longer but did not reconstitute postcephalic region (Figs. 4, 5).

When a piece of the ganglionic region of the head above certain size (Figs. 1, 2, 3) is transplanted into the prepharyngeal region very near the head of the host, it reconstitutes a head of some sort which may later be resorbed, or may become detached, or in case the host is beheaded for the second time, it may develop as in Figs. 6, 7, and 8, with very little or no outgrowth from the host tissue. And if the host is beheaded for the second or third time after transplantation, the grafted head usually decreases the head frequency of the host. If the cut in the second or third beheading is very close to the region of the graft, head formation at the cut surface may be completely inhibited; and in this case, the grafted head acts as the head of the host (Fig. 9). If the inhibition of head formation at the cut surface at the third beheading is not complete, the two heads may approach each other and sooner or later fuse together (Fig. 10).

A piece of the ganglionic region of the head (Figs. 1, 2, 3), if not too small, when transplanted into the prepharyngeal region, is not resorbed, but is capable of reconstituting a complete head and of inducing the development of an outgrowth (Figs. 11–15), and thus a new axis is formed with the grafted piece at its tip.

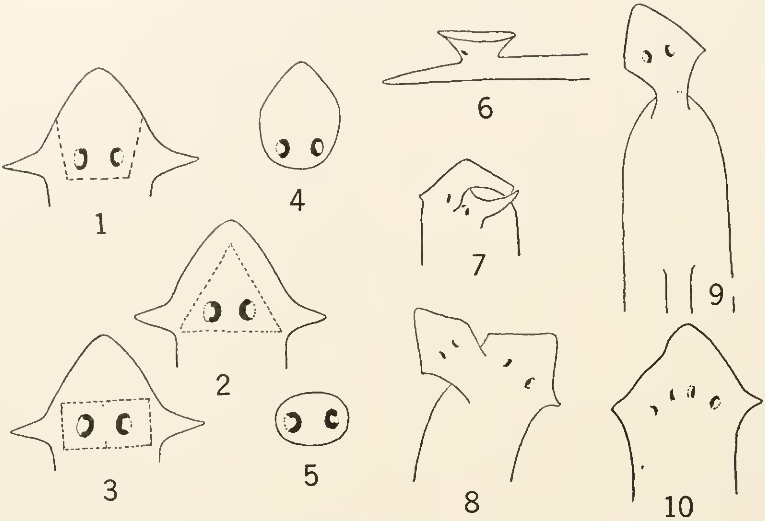
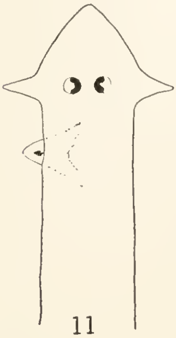


Fig. 1. Head of *Planaria* showing the part transplanted.
Fig. 2. Head of *Planaria* showing the part transplanted.
Fig. 3. Head of *Planaria* showing the part transplanted.
Fig. 4. Isolated portion of the head of *Planaria dorotocephala,* 12 days after isolation.
Fig. 5. Isolated portion of the head of *Planaria dorotocephala,* 12 days after isolation.
Fig. 6. Side view of *Planaria dorotocephala* with headgraft.
Fig. 7. *Planaria dorotocephala* with head-graft.
Fig. 8. *Planaria dorotocephala* with head-graft.
Fig. 9. *Planaria dorotocephala* with head-graft from *P. maculata.*
Fig. 10. *Planaria dorotocephala* with grafted head from *P. dorotocephala* fused with the regenerated head of the host, after the third beheading.

A piece of the ganglionic region of the head transplanted to a region very near the head of the host does not act as an organizer. When transplanted to more posterior regions, if not resorbed, it acts as an organizer. When transplanted into the postpharyngeal region near the tail, if not resorbed, it is capable of inducing the formation of a secondary pharynx in the posterior region, and of reversing the polarity of the region originally anterior to the level of the graft. This reversal of polarity is shown by the development of a new pharynx opposite in direction to the original pharynx and by complete reorganization of the alimentary tract. It may also inhibit head formation at the cut surface several millimeters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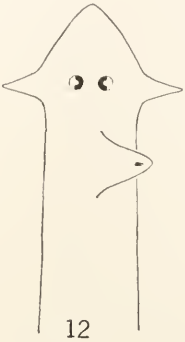
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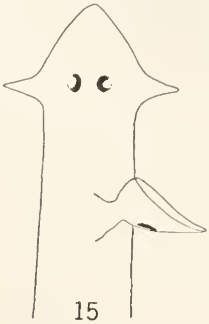
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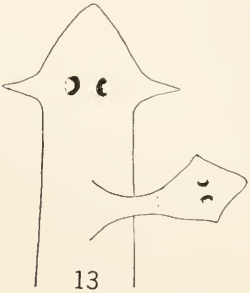
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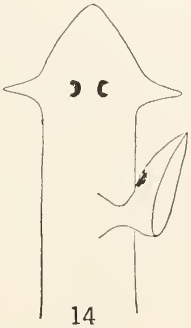
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FIG. 11. *P. dorotocephala* with head-graft developing on the ventral surface of the host. Graft is partly inhibited.

FIG. 12. *P. dorotocephala* with head-graft developing on the dorsal surface of the host. Graft is partly inhibited.

FIG. 13. *P. maculata* with head-graft from *P. dorotocephala.*

FIG. 14. *P. maculata* with head-graft from *P. dorotocephala.*

FIG. 15. *P. maculata* with head-graft from *P. dorotocephala.*

anterior to the graft after the anterior part of the host is removed for the second, third or fourth time, posterior or through the pharynx. The result in these cases apparently depends upon the physiological activity of the level of the body of the host and of

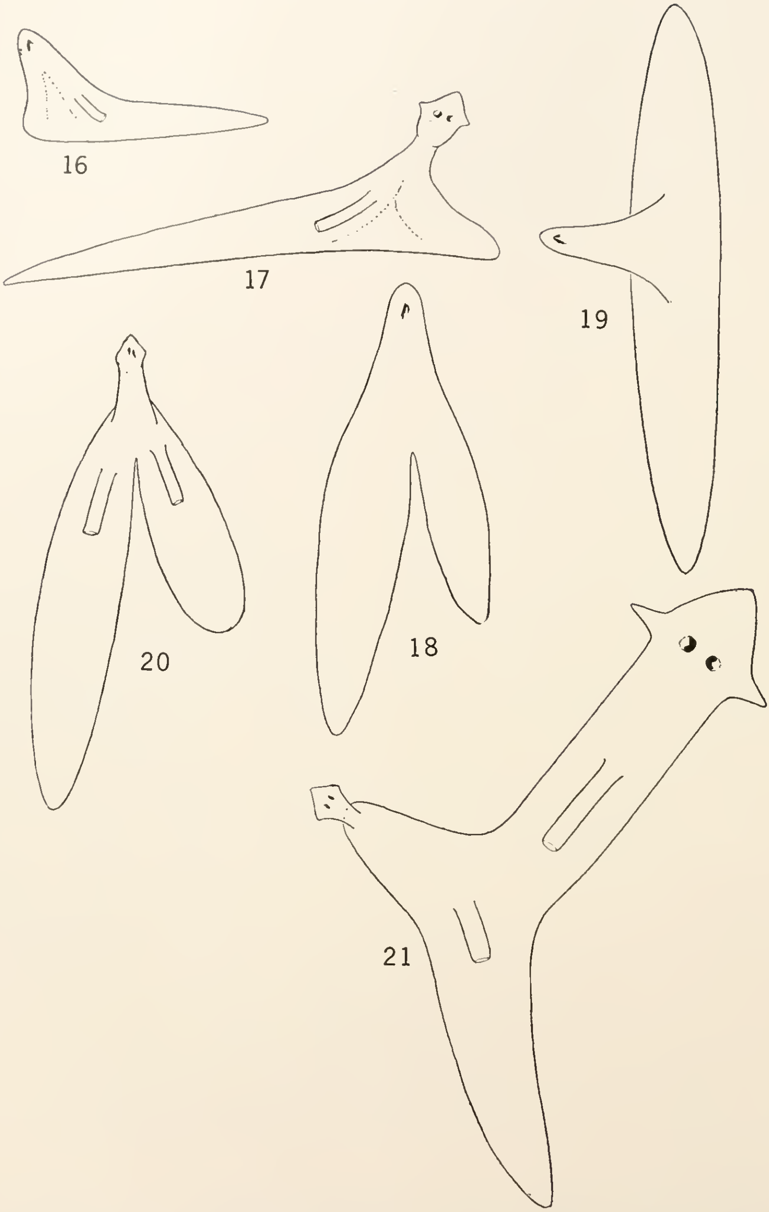
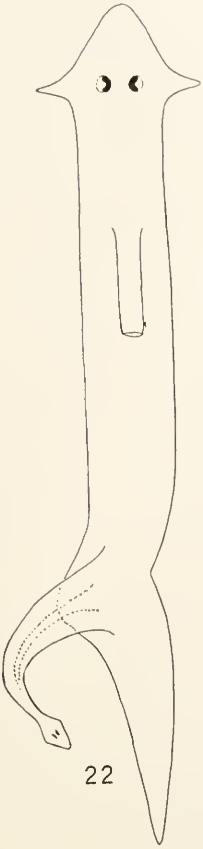


FIG. 16. *P. dorotocephala* with postpharyngeal head-graft from *P. dorotocephala.* Side view showing the induced pharynx.

FIG. 17. *P. dorotocephala* with postpharyngeal head-graft from *P. dorotocephala.* Side view showing the induced pharynx.

FIG. 18. *P. dorotocephcla* with postpharyngeal head-graft from P. *doro-*



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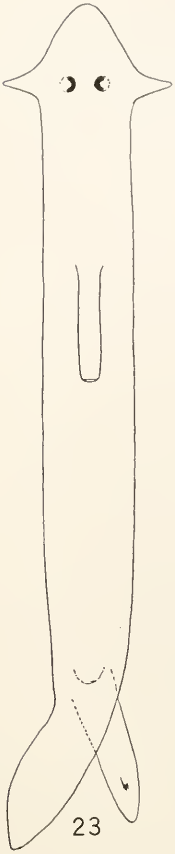

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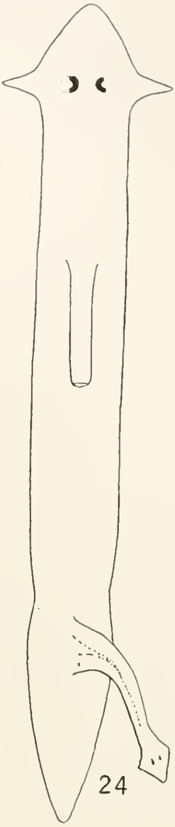

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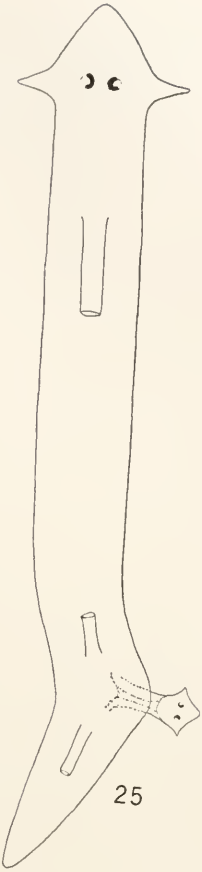

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tocephala. Regeneration of head at the anterior cut surface is prevented, and showing reversal of polarity.

Fig. 19. *P. dorotocephala* with postpharyngeal head-graft from *P. dorotocephala.* Regeneration of head at the anterior cut surface is prevented, and showing reversal of polarity.

Fig. 20. *P. dorotocephala* with postpharyngeal head-graft from *P. dorotocephala.* Regeneration of head at the anterior cut surface is prevented, and showing induced secondary pharynges and reversal of polarity.

Fig. 21. *P. maculata* with postpharyngeal head-graft from *P. dorotocephala.* Showing the induced secondary pharynx.

Fig. 22. *P. dorotocephala* with postpharyngeal head-graft from *P. dorotocephala,* showing the induced outgrowth from host tissue.

Fig. 23. *P. dorotocephala* with postpharyngeal head-graft from *P. maculata.*

Fig. 24. *P. dorotocephala* with postpharyngeal head-graft from *P. maculata.*

Fig. 25. *P. maculata* with postpharyngeal head-graft from *P. maculata,* showing the two induced pharynges and reversal of polarity.

the grafted head and of the species used as host. Cross transplantation between *Planaria maculata* and *P. dorotocephala* shows that this capacity to reverse the polarity and to induce the development of secondary pharynges in the posterior region of the host is not species-specific, for the head of *P. dorotocephala* is capable of inhibiting head formation at the cut surface several millimeters anterior to it, reversing the polarity of the region anterior to it, and inducing the formation of secondary pharynges (Figs. 16–31)

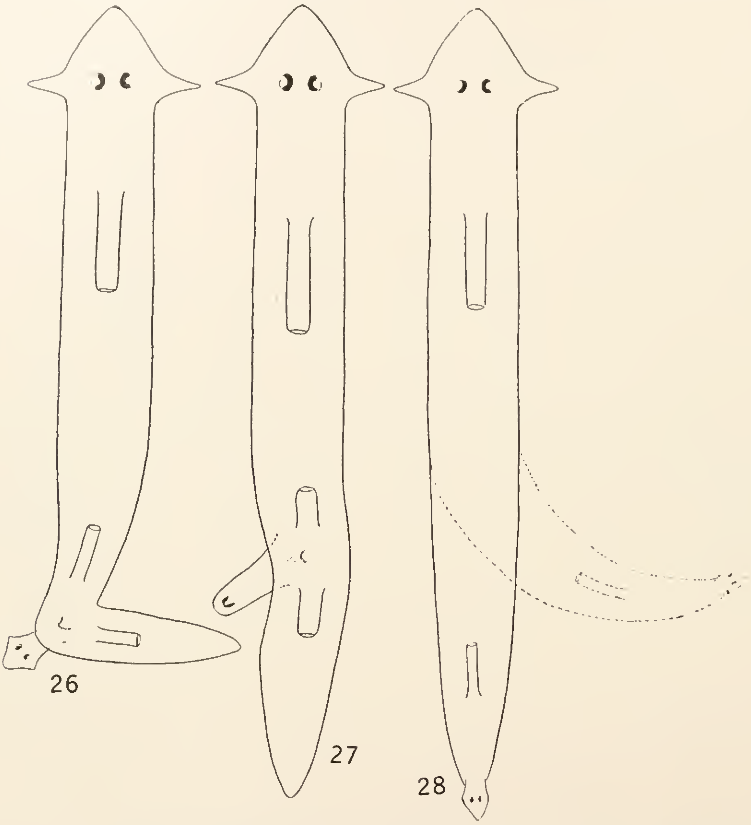

Fig. 26. *P. dorotocephala* with postpharyngeal head-graft from *P. maculata,* showing the induced pharynges and reversal of polarity.

Fig. 27. *P. maculata* with head-graft from *P. maculata,* showing the induced secondary pharynges and reversal of polarity.

Fig. 28. *P. dorotocephala* with head-graft from *P. maculata,* showing the induced pharynx and reversal of polarity.

in *P. maculata* as host and vice versa. Feeding experiments show that these secondary pharynges induced by the grafted head, are functional, *i.e.,* they are extruded and attached to the piece of liver and show the normal peristalsis of the pharynx characteristic of the feeding reaction of *Planaria.*

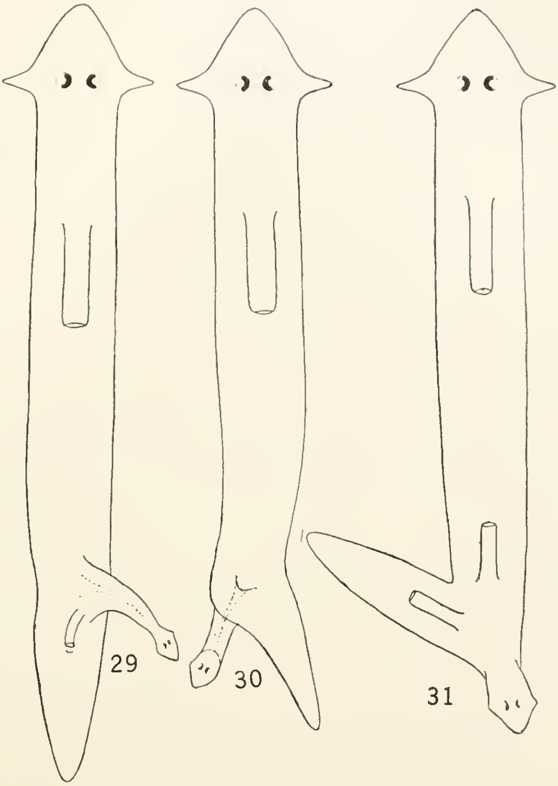

FIG. 29. *P. dorotocephala* with head-graft from *P. maculata,* showing the induced secondary pharynx and outgrowth from host tissue.

FIG. 30. *P. dorotocephala* with head-graft from *P. dorotocephala,* showing outgrowth from host tissue.

FIG. 31. *P. dorotocephala* with head-graft from *P. dorotocephala,* showing induced secondary pharynges and reversal of polarity.

Discussion.

Considering the results of all the experiments performed not merely of those herein reported, the writer is of the opinion that the fate of the graft does not depend primarily upon its degree of specialization, but rather upon the degree of physiological activity of the graft, and of the region of the host into which the piece is implanted, *i.e.*, if the degree of physiological activity of the graft is very high with respect to the region of the host where it is grafted, the graft may not be resorbed, particularly when it is far from the dominant region of the host. For example, when a portion of the head (an example of a piece of the body with a very high degree of physiological activity) is transplanted into a region very near the head of the host, the activity of the grafted head may be decreased by the dominant head of the host and it may finally be resorbed, or else fusion of the two heads may take place.

In *Planaria,* the head is the most active and hence the most dominant region of the body. This fact has been experimentally demonstrated, qualitatively and quantitatively, by Child and confirmed by others in different species of animals besides *Planaria dorotocephala.* Rand and Ellis (1926) working on *P. maculata,* had confirmed the dominance of the head. They termed it "inhibitory dominance." Rand and Browne (1926) working on *P. maculata* had demonstrated that the presence of a grafted head may inhibit the regeneration of a head at an exposed anterior cut surface. The results of some of the experiments herein reported confirm this finding of Rand and Browne.

The head of some sort developed from a graft consisting of a portion of the ganglionic region may not only inhibit the development of a head at a cut surface some distance anterior to it, but may completely reverse the polarity of a region anterior to its level and may induce the reorganization of a region posterior to its level with the formation of a secondary pharynx. Evidently the graft from the ganglionic region is able to act as an "organizer" or more strictly speaking, as a "reorganizer" (Child, 1929) of other regions of the body into which it is implanted.

Grafts of other regions of the body may in some cases give rise to outgrowths. Whether or not such outgrowth develop in particular cases depends on various factors, *e.g.*, size and orientation of graft, level from which it is taken and region into which it is implanted. Further investigation is necessary, however, to determine to what extent a real reorganization occurs in such outgrowths.

SUMMARY.

1. A new technique of transplantation in *Planaria* which gives a high per cent. of "takes" is described.

2. The head, or part of it, which is the most active region of the body of *Planaria*, when trannsplanted into the postpharyngeal region or relatively less active region of the body, is capable, not only of acting as an organizer, and completely reversing the polarity of the region anterior to it, but also inhibiting the formation of a head at a cut surface several millimeters anterior to it.

3. Both homoioplastic and heteroplastic transplantations were performed in *Planaria dorotocephala* and *P. maculata*. The results of the experiments on heteroplastic transplantation between the two species used, show that the action of the graft is not species-specific.

4. The results of the experiments indicate that the fate of the graft does not depend primarily upon the degree of its specialization but on the degree of physiological activity of the level of the body from which it is taken and also on the region of the body of the host in which it is implanted.

5. The fate of the graft also depends upon the size of the graft, degree of "take," and orientation of the graft in the body of the host.

LITERATURE CITED.

Child, C. M.
'24 Physiological Foundation of Behavior. (New York), Chap. X.

Child, C. M.
'29 Lateral Grafts and Incisions as Organizers in the Hydroid, Corymorpha. Physiol. Zoöl., Vol. II., pp. 342–374.

Rand, Herbert W., and Amy Browne.
'26 Inhibition of Regeneration in Planarias by Grafting: Technique of Grafting. Proc. Nat. Aca. Sc., Vol. 12, p. 575.

Rand, H. W., and Mildred Ellis.
'26 Inhibition of Regeneration in Two-Headed or Two-Tailed Planarians. Proc. Nat. Aca. Sc., Vol. 12, p. 570.

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BIOLOGICAL BULLETIN

THE REACTIONS OF *PARAMECIUM* TO SOLUTIONS OF KNOWN HYDROGEN ION CONCENTRATION.[1]

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Introduction.

The reactions of unicellular organisms to acids in dilute solution has been a subject for research for many years. Pfeffer in 1888 observed the positive reaction of spermatozoa to malic acid. Jennings ('97, '99*a*, '99*c*, '00*a*, '00*b*, '00*c*, '02, '06) described the attracting action of dilute acids for *Paramecium* and other protozoa. He expressed quantitative results in terms of fractional normality of acids rather than concentration of hydrogen ions. Garrey ('00), a student of Loeb, studying the reactions of *Chilomonas* to acids recognized the necessity for measuring the results in terms of hydrogen ion concentration but was able to do it only relatively rather than absolutely. Garrey and Jennings achieved results at variance and became involved in a lively controversy over the interpretations—a part of the larger Loeb-Jennings controversy. Greeley ('04) obtained positive reactions to dilute acid solutions with *Paramecia* taken from acid cultures. He states that he did not obtain positive reactions with *Paramecia* taken from alkaline cultures.

This field seems to have been abandoned by later workers. With the discovery of methods for determining the true hydrogen ion concentration of fluids and materials in recent years numerous investigations have been made concerning the part of acidity and alkalinity in biological processes, but none of these investigations have been along the lines followed by Jennings, Garrey and Greeley. Later workers (Fine, '12, Collett, '19, Kostir, '21, Bodine, '21, Saunders, '24, Hopkins, '26, Pruthi, '27, Beers, '27, Eddy, '28, Darby, '29) have been more concerned with the tolerance of organisms to acids, the toxicity of acids in the media, the changes in natural acidity in protozoan cultures, and the effects of hydrogen ion concentration on different life processes.

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[1] The writer is greatly indebted to Dr. W. C. Allee for suggesting this investigation and for facilities and advice afforded in connection with it and to Mr. M. R. Garner for his coöperation in the initial experiments.

No account of a repetition of the experiments performed by Jennings, Garrey and Greeley using modern methods of determining hydrogen ion concentration, as worked out by Sorensen and Clark and Lubs, has been found in the literature. The series of experiments described in this paper was undertaken to determine the reactions of *Paramecia* to acid solutions of known hydrogen ion concentration. The experiments were started during the summer of 1927 [1] in Whitman Laboratory and were continued there during the summers of 1928–29. During the winters of 1927–28 and 1928–29 the work was carried on at Wabash College, Crawfordsville, Indiana.

Methods and Materials.

A preliminary study was made to find satisfactory methods for carrying on the main series of experiments. Pfeffer (fide Loeb, '18) introduced the open end of a capillary tube sealed at the other end and filled with acid solution, into a drop of organisms. Loeb ('18) observes that Pfeffer's method of testing chemotropism is the best. Barratt (fide Loeb, '18) concluded, using Pfeffer's capillary tubes as described above, that *Paramecia* were indifferent to acid solutions. Barratt's experiments were repeated using HCl (pH 5.2) in capillary tubes and like results were obtained. When slightly larger tubes were used the organisms entered the acid in about ten times the numbers that entered the control. Ordinary pipettes were used with the result that seventy-five animals entered the acid while one entered the control. From these results it seemed evident that in the case of very small tubes it is almost impossible for a *Paramecium* swimming spirally to enter without striking the edge of the opening. And so Pfeffer's capillary tubes were not considered practical in these experiments. Jennings ('06) placed a cover slip, slightly elevated with glass rods, on a slide; introduced a drop of medium containing organisms on one side and acid solution on the other by means of a capillary pipette, or, merely placed a bit of acid at the edge of a cover slip under which the organisms lay. Garrey ('00) used a hollow cell for holding the organisms and introduced the acid through a small opening on one side. In addition to these methods two others were tried. (1) An acid gradient was made in a long tube (24 inches); the organisms were introduced at the end of the gradient corresponding in H-ion concentration to their own medium, and (2) a "double drop" method was devised in which a drop of medium containing *Paramecia* and a drop of acid solution were placed side by side on a slide and the two drops were connected by leading the liquids toward each other in a narrow line with the point of a needle. The former of these methods was discarded as being utterly impractical and the latter was chosen for use in preference to the older methods for a number of reasons. (1) Less elaborate apparatus was required. (2) The experiments were set up and carried on more quickly. (3) A more gradual gradient could be established than with the older methods.

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[1] Mr. M. R. Garner and the present writer worked together on the first set of experiments to be described.

Trays were made for holding the slides during the experiments. These trays, made by Mr. Carson in the shop at Whitman Laboratory, were fifteen inches in length, four inches in width and two and a half inches in depth with grooves on the sides to hold the slides in place. It was found that strips of black paper placed in the bottom of the trays aided greatly in making the counts at the beginning and end of each experiment.

The colorimetric method of determining hydrogen ion concentration was used except as noted. The standard tubes and indicators used were purchased from the La Motte Chemical Products Company or the Hynson, Westcott and Dunning Company, both of Baltimore, Md. The standard tubes covered a range from pH 3.0 to pH 8.4. The indicators were brom phenol blue, brom cresol green, methyl red, brom cresol purple and phenol red. The indicators, with the exception of methyl red, were made up in aqueous solutions. The exception was made up in an alcoholic solution as it is only slightly soluble in water, but the quantity of alcohol introduced into a solution when this indicator was used was far below what Jennings found to elicit any sort of response. Both methyl red and brom cresol green were used in determining pH 5.0. Neither seemed to give very accurate and reliable results because the color change at this point is not very marked in the case of both. Hyman ('25) has noted this same difficulty with brom cresol green. Due to this difficulty it seems possible to explain some of the inconsistencies in the data at H-ion concentrations of pH 5.0.

Preliminary experiments were made to determine the degree of diffusion between the two drops. The usual result is illustrated in Fig. 1. Complete diffusion required about an hour while the experiments usually ran about half an hour. The culture drops start drying first so any reading taken after half an hour would likely be affected by the drying in the culture drop. It was found that if the slides were too clean the drops spread rather badly. This could usually be remedied by rubbing one's hand over the slide to coat it with a film of oil. Experiments were carried on in duplicate, *i.e.* two slides bore the same drop combination. Experiments rendered unsatisfactory on account of the drops spreading or for other reasons were discarded. It was determined that it made no difference if the indicator was included in the acid solution.

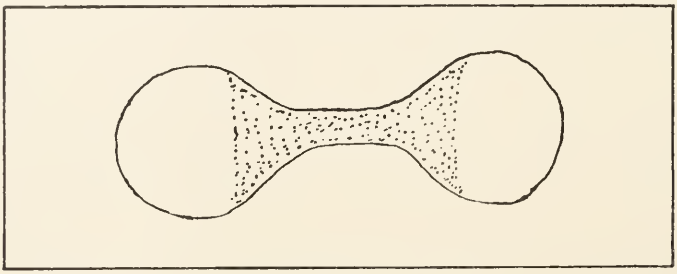

FIG. 1.

Hay infusions were prepared by boiling 5 grams of timothy hay in 500 cc. of water for about five minutes. An additional 500 cc. of unboiled water was added to the infusion and after it had stood for a few days it was seeded with *Paramecia*. *Paramecium caudatum* as described by Wenrich ('28) were used.

The original seed was obtained from Dr. Peterson in Whitman Laboratory and all additional cultures were seeded from this strain. The cultures were not used until they were two or three weeks old. A new culture was prepared each week so that a number of cultures were on hand throughout the experiments. At first the cultures were kept in 1000 cc. Erlenmeyer flasks. It was found that the H-ion concentration of such cultures remained high for a long time. The cultures for practically all of the experiments were kept in wide-mouthed bottles and finger bowls. According to Jennings ('06), the fact that *Paramecia* collect in cultures in clumps where carbon dioxide is present often causes misleading results, so in all of the experiments the *Paramecia* were stirred and aërated to eliminate this factor.

The following acids were used: hydrochloric, nitric, sulphuric, carbonic, formic, acetic, citric, picric, pyrogallic, and tannic. Carbonic acid was prepared by bubbling CO_2 into water until the desired H-ion concentration was obtained. As the CO_2 soon escapes into the air and thus changes the H-ion concentration, it seemed necessary to find a way of covering the drops when carbonic acid was used. This was done by cementing glass strips on the edges of ordinary slides. The tray holding these "chamber slides" was placed in a large covered pan and CO_2 was allowed to escape into the pan. Then the drops were placed in the "chamber slides" and connected as quickly as possible. As soon as the drops were connected each "chamber slide" was covered with an ordinary slide. Indicators showed that the change in H-ion concentration from the time the drop was placed on the slide until it was covered was not great.

The following different kinds of water were used in making up the acid solutions: ordinary Chicago tap water, Hull[1] well water, Whitman[2] well water, Crawfordsville (Ind.) tap water, distilled water, Chicago tap water made carbonate-free, Whitman well water made carbonate-free and Hull well water made carbonate-free. The carbonate-free water was made according to the method used by Hyman ('25). Two cc. of concentrated

[1] From Hull Zoölogical Laboratory.

[2] From Whitman Experimental Laboratory.

hydrochloric acid were used to eight liters of water in a large bottle. Air from the compressed air system was passed through the water in the bottle for at least 24 hours. Analyses of the Whitman well water and the Hull well water will appear later in the paper.

At first there was a question as to what constituted a positive reaction. The constant moving about of the *Paramecia* made it appear that some of the crossing over from one drop to another must be attributed to random movements on the part of the *Paramecia*. It was thought that the absence of *Paramecia* from one drop might be a factor in causing the crossing over. A set-up was made to test this idea. Some culture liquid from a culture with a concentration of pH 7.7 was centrifuged. The culture fluid, free from *Paramecia*, was drawn off and used in an experiment in the same way that acidified water had been used—a drop of the culture fluid free from *Paramecia* was joined to a drop of culture containing *Paramecia*. There was no change in the H-ion concentration from centrifuging. Table I gives the result of this experiment. At the time of reading which was that used in other experiments about equal numbers were found in each drop. On the basis of these results, reactions of over 55 per cent were considered as positive reactions. This indicates that the absence of *Paramecia* from one of the drops is not a factor in causing the positive reactions.

Table I.

Showing Amount of Random Movement on Part of *Paramecia*.

	pH 7.7 (Centrifuged Culture liquid)					
	45	55	50	51	46	48
	46	46	56	60	51	73
	53	58	41	49	56	52
	36	61	40	40	53	43
	56	56				26/1326
Ave. % reaction.						51

A number of experiments were undertaken during the Christmas holidays (1928–29) but not many were successful. The temperature of the laboratory during this time ranged from 15° C.

to 21° C. The only experiments which gave any positive results were conducted when the temperature was 21° C. The experiments run when the temperature was lower than 21° C. gave no positive results. Jennings ('06) states that Mendelssohn found that the optimum temperature for *Paramecium* is between 24 and 28 degrees C. All of the experiments recorded here were conducted within a temperature range of 21 to 28 degrees C. No data were obtained as to the upper temperature limit for positive reactions.

EXPERIMENTS.

(1) *To Determine pH Range in which Positive Reactions Occur.*

In the beginning several experiments were carried out using a pH range of 3.2 through 5.8. It became evident, after using several acids and repeating the experiments, that no positive reaction (it was considered that the *Paramecia* must enter the acid drop) could be obtained at a H-ion concentration greater than pH 5.0 with any of the acids used. Any H-ion concentration of pH 4.8 or lower proved to be toxic to *Paramecium.* So with the lower limit for positive reactions established, the next problem was to determine the reactions of *Paramecium* to a range of H-ion concentrations of pH 4.8–5.8. The following table shows the results obtained with hydrochloric, sulphuric and nitric acids in Chicago tap water.

Section *A* of this table shows the results of six set-ups. Two slides with the same H-ion concentration were used in each set-up, as the table shows. The numbers in the different columns represent the percentage reaction on each slide from which the mean average reaction was obtained. The number of *Paramecia* on each slide averaged about fifty. This was true for all of the experiments. According to the criterion established above Table II. shows positive reactions from pH 5.0 through pH 5.8 in the three inorganic acids used. There seemed to be no significant difference in the reactions with the different acids. The H-ion concentration of the cultures used in these experiments ranged from pH 7.4 to pH 8.0. Controls (in duplicate) were run in each set-up by joining a drop of culture with a drop of unaltered tap water. No positive reaction occurred in any of the controls. The high percentage reaction (81 per cent.) at

Table II.

Showing the Percentage Reactions of *Paramecia* to Hydrogen Ion Concentrations of pH 4.8–5.8 Made with Hydrochloric, Sulphuric and Nitric Acids in Chicago Tap Water.

(Data in section *A* obtained in conjunction with Mr. M. R. Garner.)

A (1927).

	pH 4.8	5.0	5.2	5.4	5.6	5.8
Hydrochloric	10	73	96	84	80	22
	12	84	70	73	88	25
Hydrochloric	25	18	70	90	67	78
	10	27	92	80	50	79
Sulphuric	23	23	95	70	72	76
	4	42	89	71	70	69
Sulphuric	41	56	59	54	43	74
	58	87	74	55	39	18
Nitric	60	68	87	55	56	61
	45	88	65	65	60	51
Nitric	22	65	82	75	52	50
	58	56	92	59	83	58
	12/368	12/686	12/971	12/829	12/760	12/661
Ave. % reactions	30	57	81	69	63	55

B (1928).

	pH 4.8	5.0	5.2	5.4	5.6	5.8	7.8
Hydrochloric	35	73	53	67	80	91	40
	70	83	54	59	89	94	
Hydrochloric	23	41	43	47	91	70	6
	50	47	77	37	97	78	
Hydrochloric	44	65	54	80	23	30	9
	20	63	95	30	56	63	
Hydrochloric	36	53	81	70	74	71	26
	52	76	92	70	100	97	
Sulphuric	44	65	92	54	72	98	41
	60	40	80	51	78	75	
Sulphuric	41	72	67	97	67	67	9
	58	50	83	54	63	72	
Sulphuric	9	70	74	77	60	69	30
	34	74	70	54	50	85	
Sulphuric	15	90	85	72	83	64	21
	17	80	77	72	77	61	
Nitric	13	70	67	82	70	60	34
	22	81	67	82	52	87	
Nitric	18	50	73	62	80	77	0
	20	82	70	71	80	65	
Nitric	28	82	50	74	88	70	8
	29	80	60	81	80	68	
	22/738	22/1487	22/1564	22/1463	22/1610	22/1611	11/224
Ave. % reactions	33	67	71	66	73	73	20

pH 5.2 seemed to be a possible optimum but later experiments did not indicate such a marked reaction to any one H-ion concentration. In all of the experiments the temperature of the acid solutions was maintained the same as the temperature of the culture used.

The results in Section *B* are very similar to those listed in Section *A*. One difference seems to be that there is no marked optimum in the reaction given at any pH as was the case in the reactions to a concentration of pH 5.2 in Section *A*. The figures in the column under pH 7.8 show the results in the controls which were like the controls used in the first series of experiments. The cultures used ranged in H-ion concentration from pH 7.4 through pH 7.8. Another difference is evident in that the percentage of reactions at pH 5.8 in the second series is no lower than at any other pH in the range used. Thus it became necessary to extend the range of experimentation. A greater range had not been used in the first series because time would not permit.

Several organic acids were used over the same pH range. Acetic, citric and formic acids elicited responses somewhat comparable to those of the inorganic acids as shown in Table II. Pyrogallic and tannic acids proved to have such a high degree of toxicity for the animals that reactions within the pH range studied were impossible—death resulting on contact with the weakest part of the diffusion area. This high degree of toxicity of tannic acid may offer an explanation of the absence of Protozoa from bog waters. Picric acid was used several times with inconsistent results. The strong color of the acid solution prevented accurate pH readings. It also seemed that here, as with pyrogallic and tannic acids, there is some other factor besides the H-ion concentration which affects the reactions of *Paramecia*.

Later in the summer of 1927 cultures with higher H-ion concentrations (pH 6.8, pH 7.0 and pH 7.2) were used and no positive results were obtained at the above ranges. Several times throughout the period of experimentation cultures with these concentrations were used with the same results—no positive reactions. When the H-ion concentration of the cultures fell, positive results were obtained.

Table III. shows that positive reactions can be obtained in a pH range of 6.0–6.8, which, taken with the results of the two other series, means that *Paramecia* from alkaline cultures react positively throughout a pH range of 5.0–6.8. No positive response was obtained in the controls at pH 7.8.

TABLE III.

SHOWING THE PERCENTAGE REACTIONS OF *Paramecia* TO HYDROGEN ION CONCENTRATIONS OF pH 6.0–6.8 MADE WITH HYDROCHLORIC, SULPHURIC AND NITRIC ACIDS IN CHICAGO TAP WATER.

	pH 6.0	6.2	6.4	6.6	6.8	7.8
	44	78	88	57	79	
Hydrochloric	62	66	66	65	54	38
	54	84	81	61	76	
Hydrochloric	83	85	55	70	77	2
	40	40	62	80	63	
Sulphuric	53	40	70	58	80	0
	51	48	51	58	62	
Sulphuric	33	51	36	52	57	38
	89	77	81	77	57	
Nitric	80	43	73	70	60	21
	46	48	46	43	56	
Nitric	57	49	54	50	46	30
	46	81	64	59	69	
Nitric	76	75	54	65	76	0
	14/814	14/865	14/881	14/865	14/914	7/129
Ave. % reactions	58	62	63	62	65	18

A series of experiments were run using Hull well water. The following table gives the results when unaltered Hull well water was acidified with nitric, hydrochloric and sulphuric acids.

The cultures used in Table IV. had a H-ion concentration of pH 7.8. Unaltered Hull well water, pH 7.8, was used in the controls. These data show that *Paramecia* from cultures with a low H-ion concentration (pH 7.8) react about the same way to acidified Hull well water as they react to acidified Chicago tap water.

All the data seem to point rather conclusively to the fact that *Paramecium caudatum* as reared in these cultures do not react positively to H-ion concentrations greater than pH 5.0. As one of the objects of this problem was to determine the pH range for positive reactions, it seemed necessary at this point to run more experiments attempting to determine the upper limit for positive responses. Crawfordsville (Ind.) tap water, coming from a number of surface springs, was used. This water always had a H-ion concentration of pH 7.4. Table V. gives the results of a series of experiments using unaltered Crawfordsville tap water, acidified with hydrochloric, sulphuric and nitric acids, over a pH range of 5.0 to 7.2. The unaltered tap water, pH 7.4, was used in the series as a control. The cultures used in this series had H-ion concentrations of pH 7.6 to pH 7.8.

Table IV.

Showing the Percentage Reactions of *Paramecia* to Hydrogen Ion Concentrations of pH 4.8–5.8 Made with Hydrochloric, Sulphuric and Nitric Acids in Hull Well Water.

	pH 4.8	5.0	5.2	5.4	5.6	5.8	7.8
	9	72	90	85	79	79	
Hydrochloric........	23	80	79	70	71	89	47
	20	36	60	90	93	70	
Sulphuric...........	15	20	50	83	79	65	43
	10	40	70	84	80	79	
Nitric..............	17	30	81	89	89	76	26
	6/94	6/278	6/430	6/501	6/489	6/458	3/116
Ave. % reactions....	16	46	72	83	81	76	38

It will be noticed by examining Table V. that positive reactions were obtained throughout the entire range used—pH 5.0–7.2. And not only were positive reactions obtained throughout this range, pH 5.0–7.2, in which the H-ion concentration was produced by adding acid to the water, but positive reactions were also obtained in the controls, unaltered tap water, with a H-ion concentration of pH 7.4. If all of the preceding tables are examined it will be found that in the other controls the H-ion concentration in each case was the same as, or lower than, the H-ion concentration of the culture used. In this case the H-ion concentration of the control used was higher than the hydrogen ion concentration of the cultures used. This pointed to another set of experiments using water with a H-ion concentration the same as the culture used. All of the controls of the preceding experiments indicate that *Paramecium* will not react positively to a solution with a H-ion concentration the same as, or lower than, the H-ion concentration of the culture. Indicators showed

TABLE V.

SHOWING THE PERCENTAGE REACTIONS OF *Paramecium* TO HYDROGEN ION CONCENTRATIONS AT pH 5.0–7.2 MADE WITH HYDROCHLORIC, SULPHURIC AND NITRIC ACIDS IN CRAWFORDSVILLE TAP WATER.

	pH 5.0	5.2	5.4	5.6	5.8	6.0	6.2	6.4	6.6	6.8	7.0	7.2	7.4
HCl..........	44	74	83	67	87	80	75	71	74	48	79	64	73
	52	65	62	70	81	81	75	74	53	70	83	83	70
HCl..........	43	68	63	86	82	81	84	70	87	74	74	91	59
	70	76	85	84	86	69	78	79	80	75	72	81	69
HCl..........	84	75	88	78	92	69	80	72	69	72	89	75	78
	63	70	85	88	81	76	64	79	85	71	86	78	73
HCl..........	51	57	72	76	81	80	65	69	67	78	73	73	76
	57	70	80	64	70	75	59	75	69	62	78	72	65
HCl..........	54	67	80	100	86	78	86	88	85	84	89	88	58
	62	80	54	86	65	57	74	79	93	71	75	88	56
HCl..........	33	55	71	83	74	72	83	56	65	82	73	84	69
	31	47	68	84	74	81	77	62	89	81	80	74	58
H_2SO_4..........	27	43	78	81	77	92	79	89	83	84	79	89	63
	32	48	86	79	84	89	88	72	87	81	88	87	56
H_2SO_4..........	62	60	81	76	77	79	80	68	77	80	80	83	82
	55	77	73	72	70	75	82	74	75	77	79	75	78
H_2SO_4..........	30	53	62	65	74	69	75	60	50	54	49	57	61
	48	24	68	50	79	75	69	60	53	40	70	46	83
H_2SO_4..........	24	92	86	91	91	92	100	89	82	81	86	83	77
	25	91	84	87	89	91	87	89	66	80	77	86	83
H_2SO_4..........	26	41	32	67	84	50	62	52	40	31	60	84	83
	27	44	32	60	62	41	55	44	46	41	66	79	87
H_2SO_4..........	45	47	75	60	75	60	71	87	72	70	76	50	61
	59	63	72	61	51	78	75	66	72	74	50	56	73
HNO_3..........	64	83	90	90	76	79	94	35	75	88	70	79	84
	53	79	81	76	81	84	85	85	72	89	90	88	82
HNO_3..........	41	53	61	53	79	70	77	84	88	81	51	53	79
	53	56	60	80	53	69	80	83	91	85	54	82	59
HNO_3..........	26	57	60	58	71	44	40	75	55	53	70	76	33
	20	56	63	61	74	46	52	63	51	67	89	64	41
HNO_3..........	59	46	81	80	71	69	73	79	63	74	86	67	76
	55	49	74	89	79	85	75	79	74	71	77	76	80
HNO_3..........	56	43	60	93	85	76	41	66	64	82	50	65	64
	58	54	93	84	70	60	38	84	73	81	68	65	70
Ave. % reactions	34/1592 47	34/2063 61	34/2443 72	34/2574 76	34/2601 76	34/2470 72	34/2478 72	34/2455 72	34/2435 71	34/2432 71	34/2516 74	34/2541 74	34/2359 69

that the H-ion concentration of the drops made from Crawfordsville tap water fell as soon as the drops were exposed to the air, so it was possible by aërating the water to lower the H-ion concentration to pH 7.8. Table VI. gives the results of the set of experiments using *Paramecia* from a culture with a concentration of pH 7.8 and Crawfordsville tap water aërated so that the concentration was pH 7.8. A control was used by testing the reaction of *Paramecia* to a concentration of pH 5.6 made by adding nitric acid to Crawfordsville tap water. Positive reactions were obtained in the controls but not in the drops where the H-ion concentration of the two drops was the same. This would seem to indicate the *Paramecia* from cultures with a low H-ion concentration react positively to any H-ion concentration higher than that of their culture to a pH of 5.0, which is the lower limit for positive reactions.

Table VI.

Showing the Percentage Reactions of *Paramecia* to a Hydrogen Ion Concentration of pH 7.8 Made by Aërating Crawfordsville Tap Water.

	5.6 (Nitric Acid).	7.8 (Aërated Crawfordsville Tap Water).				
	75	11	12	35	44	31
	82	4	18	40	28	41
	61	27	16	24	33	27
	78	38	27	24	36	11
	4/296	36	41			22/639
Ave. % reactions. .	74					29

(2) *To Determine Whether the Positive Reactions are Due to Carbon Dioxide or to Hydrogen Ions.*

Jennings ('06) suggests that the positive reactions of *Paramecia* to dilute acids is closely connected with their natural tendency to collect in clumps when a large amount of carbon dioxide is present. Hyman ('25) found that "the depressing action of acids in natural waters is due chiefly or wholly to the carbon dioxide which they liberate from the carbonates of such waters." With these ideas in mind a series of experiments was run in an attempt to determine whether the *Paramecia* react to carbon dioxide or to the free hydrogen ions. Twelve sets (like the sets described in the preceding tables) were run from pH 4.8–6.8 with carbonate-free water made from Whitman well water using hydrochloric, sulphuric and nitric acids with no positive results. I was ready to conclude from these results that *Paramecia* react to carbon dioxide and not to free hydrogen ions when I realized that Whitman well water had not been used in any of the preceding experiments. So twelve more sets were run over the same range using Whitman well water as it comes from the tap and acidified with the same acids. These results were also negative. As a result the use of Whitman well water had to be discontinued. I can give no reason for the negative results. Analyses of Whitman well water, as well as of Hull well water with which positive results were obtained, were made but the differences were not great. No presence of B. coli was found in Whitman well water; so it seems that the unfavorable reactions to this water must be due to obscure chemical causes.

Some Hull well water was then made carbonate free in the manner already described. The carbonate-free water had a pH of 4.6. The pH was raised to 7.8 by adding NaOH. The water was then acidified. Table VII. shows the results of a series of experiments using carbonate-free Hull well water acidified with hydrochloric, sulphuric, and nitric acids.

TABLE VII.

SHOWING THE PERCENTAGE REACTIONS OF *Paramecia* TO HYDROGEN ION CONCENTRATIONS OF pH 4.8–5.8 MADE WITH HYDROCHLORIC, SULPHURIC AND NITRIC ACIDS IN CARBONATE-FREE HULL WELL WATER.

	pH 4.8	5.0	5.2	5.4	5.6	5.8	7.8
Hydrochloric	48	67	53	55	89	51	7
	40	40	78	70	87	57	34
Sulphuric	39	34	55	70	83	75	3
	60	65	54	92	81	77	3
Nitric	51	66	54	56	86	79	27
	30	60	54	75	94	73	23
Ave. % reactions	6/268	6/332	6/348	6/418	6/520	6/412	6/97
	45	55	58	69	86	69	16

The cultures used in the above experiments had *a* concentration of pH 7.6. Carbonate-free Hull well water with a sufficient amount of sodium hydroxide to make the pH 7.8 was used in the controls. This series of experiments indicated that the *Paramecia* were reacting to the free hydrogen ions and not to the carbon dioxide.

The reaction of *Paramecia* to Hull carbonate-free well water acidified with carbonic acid was tried. The carbonate-free water was lowered to pH 7.8 with sodium hydroxide. Carbon dioxide was then passed through the water until the desired acidity (pH 5.6) was obtained. It was found that carbonic acid elicited a response similar to that elicited by the three inorganic acids. The experiment was carried out in the manner described earlier in the paper. Table VIII. shows the results. The cultures used had H-ion concentrations of pH 7.7 and pH 7.8. Carbonate-free water with sodium hydroxide added to it giving a H-ion concentration of pH 7.8 was used in the controls.

Table VIII.

Showing the Percentage Reactions of *Paramecia* to a Hydrogen Ion Concentration of pH 5.6 Made with Carbonic Acid in Carbonate-free Hull Well Water.

	pH 5.6	7.8
	76	40
Carbonic acid..................	71	20
" "	94	25
" "	88	25
" "	76	0
" "	73	20
" "	75	45
" "	50	20
" "	73	11
" "	12	21
" "	44	37
" "	64	10
" "	89	4
" "	70	3
" "	78	20
" "	67	9
" "	83	11
" "	70	21
	18/1253	18/342
Ave. % reactions................	70	19

Chicago tap-water was made carbonate-free in the same manner and used in a series of experiments. The Chicago carbonate-free tap-water tested pH 4.6 when it was made, and, as in the experiments with Hull carbonate-free well water, the H-ion concentration was lowered to pH 7.8 by adding sodium hydroxide. Table IX. gives the results of a series of experiments using Chicago carbonate-free tap-water. The cultures used tested pH 7.8. Carbonate-free water plus sodium hydroxide with a H-ion concentration of pH 7.8 was used in the controls. The results are like those obtained in the other experiments over this pH range except in the cases where Whitman well water was used. These results also indicate that the *Paramecia* react to free hydrogen ions and not to carbon dioxide.

Table IX.

Showing the Percentage Reactions of *Paramecia* to Hydrogen Ion Concentrations of pH 4.8–5.8 Made with Hydrochloric, Sulphuric and Nitric Acids in Carbonate-free Chicago Tap-Water.

	pH 4.8	5.0	5.2	5.4	5.6	5.8	7.8
Hydrochloric	53	86	52	72	86	86	21
	55	33	88	61	76	63	46
Sulphuric	46	63	74	40	83	65	21
	30	64	76	83	81	46	42
Nitric	42	44	61	85	86	66	22
	20	41	62	77	75	64	16
Ave. % reactions	6/246	6/331	6/413	6/418	6/487	6/390	6/168
	41	55	69	69	81	65	28

(3) *To Determine Reactions of Paramecia to Distilled Water.*

Two sets of experiments were run using distilled water. The distilled water tested pH 5.6 and the *Paramecia* reacted to it positively. However, in order to run a series of experiments over the pH range 4.8 to 5.8, the H-ion concentration of the distilled water was lowered to pH 7.8 by adding NaOH. The water was then acidified to obtain the desired H-ion concentrations. The cultures used had a H-ion concentration of pH 7.7–7.8. Distilled water plus NaOH with a H-ion concentration of pH 7.8 was used in the controls. Table X. gives the results of two sets of experiments using distilled water altered with NaOH. Positive results were obtained but these results differed from any of the other results obtained using inorganic acids in this respect; in all of the drops the *Paramecia* were dead, or at least becoming inactive, at the time of the reading.

TABLE X.

SHOWING THE PERCENTAGE REACTIONS OF *Paramecia* TO HYDROGEN ION CONCENTRATIONS OF pH 4.8–5.8 MADE WITH HYDROCHLORIC AND NITRIC ACIDS IN DISTILLED WATER.

	pH 4.8	5.0	5.2	5.4	5.6	5.8	7.8
	13	67	65	56	61	100	10
Hydrochloric........	60	54	60	42	63	62	7
Nitric..............	25	82	77	63	75	54	27
	37	88	69	77	60	76	25
	4/135	4/291	4/261	4/238	4/259	4/292	4/69
Ave. % reactions....	34	73	65	60	65	73	17

(4) *To Determine pH at the End of the Experiments.*

It was evident that the H-ion concentration in the drops fell during the duration of the experiments. An attempt was made to determine the H-ion concentration at the end of the experiments. The sulphonphthalein indicators apparently are not harmful to *Paramecia* and do not affect their reactions when no greater concentration of the indicator is used than is necessary for making a pH reading in one of the tubes. Indicators were added to the drops used in the experiments. The results of many observations are given in Table XI. It is very difficult to make pH readings of the drops at the end of the experiment in this way. The density of the color in the drops is much less than the density of the color in the tube when the pH reading is determined. If enough of the indicator is added so that the density of the color in the drops is like that in the standard tubes, it is impossible to make accurate pH readings of the liquid to start with. So these observations recorded in Table XI. must be considered only as approximations. The writer is not convinced that the H-ion concentration at the end of the experiment is of very great importance because in many cases quite positive reactions occurred before the H-ion concentration had changed to any appreciable degree.

(5) *To Compare Determinations Made with Indicators and with Potentiometer.*

All of the H-ion concentration determinations which have been listed above were made using the colorimetric method. An additional series of experiments was run in which the H-ion concentrations were determined by both the colorimetric method and the electrometric method for the sake of comparison. A Youden hydrogen ion concentration apparatus, manufactured by the W. M. Welch Co. of Chicago, was used in making the electrometric determinations. The H-ion concentrations determined with the potentiometer were consistently lower than those made using the color indicators, showing a difference in pH of .15 to .26 with a mean difference of .21. The reactions of *Paramecium*

Table XI.

Showing pH at Start and End of Experiments.

pH at Start of Experiment.	pH at End of Experiment.
5.0	5.8 to 6.2
5.2	6.4 to 6.6
5.4	6.8 to 7.2
5.6	7.2
5.8	7.4
6.0	7.4
6.2	7.6
6.4	7.6
6.6	7.6
6.8	7.6
7.0	7.8
7.2	7.8
7.4	7.8

Table XII.

Showing the Percentage Reactions of *Paramecia* to Hydrogen Ion Concentrations of pH 5.0–7.6 Made with Nitric Acid in Chicago Tap-Water and the Difference in Hydrogen Ion Concentrations when Measured with Both Color Indicators and Potentiometer.

pH Color.	pH Potentiometer.	Difference.	Reactions.
5.0	4.97	0.21	56
5.2	5.00	0.20	73
5.4	5.16	0.24	81
5.6	5.38	0.22	81
5.8	5.62	0.18	79
6.0	5.79	0.21	79
6.2	5.96	0.24	78
6.4	6.21	0.19	77
6.6	6.38	0.22	71
6.8	6.65	0.15	74
7.0	6.79	0.21	78
7.2	7.03	0.17	77
7.4	7.20	0.20	73
7.6	7.36	0.24	66
7.8 (tap water)	7.55	0.25	31
7.7 (culture)	7.44	0.26	

were about the same as those in the other experiments. The following table will show the results. The figures listed under the column "pH Potentiometer" represent the average of three determinations. The figures under the column "Difference" represent the average difference of the three determinations. The figures under the column "Reactions" represent the average reactions of five observations at each H-ion concentration.

It should be stated that the above comparison does not affect the results of the other experiments. This comparison merely shows that the pH range is moved back approximately 0.21 when the H-ion concentration is determined with the potentiometer used in this case. The almost identical mean determinations (4.97 and 5.00) with the potentiometer indicates the difficulty mentioned before in determining H-ion concentrations of pH 5.0 and pH 5.2 with the color indicators.

DISCUSSION.

Jennings ('06) says that "specimens in water that is decidedly alkaline collect even more readily in acids than do those in a neutral fluid." This was found to be the case in these experiments except that *Paramecia* from slightly acid and neutral cultures did not give positive responses according to the method used in reckoning positive responses. Greely ('04) states that only *Paramecia* from acid cultures react positively to acids. This does not agree with Jennings. There is nothing in these experiments which is in agreement with this statement; in fact I have found the exact opposite to be true.

Child and Deviney ('26), Pruthi ('27) and many others have mentioned the different stages that occur in the life history of a protozoan culture. Four distinct stages were noticed in the cultures of *Paramecia* used in these experiments. The first stage was not very long in duration except in cases when Erlenmeyer flasks were used, and the H-ion concentration was high (pH 6.8–7.0). The second stage was marked by a steady fall in H-ion concentration. In the third stage the H-ion concentration was low and remained fairly constant. In this stage the *Paramecia* were scattered throughout the culture. Most of the *Paramecia* used in these experiments were in this stage. The last stage was marked by the *Paramecia* gathering in the bottom of the culture and dying out. *Paramecia* from cultures in the last stage were never used in the experiments because it seemed that the toxic substances in such a culture, as well as the general physiological state of the *Paramecia*, might prevent normal reactions.

An examination of all of the data shows that *Paramecium* (from cultures with a low H-ion concentration) react positively to all H-ion concentrations higher than that of their culture to a concentration of pH 5.0 inclusive. This limit for positive reactions is the same as Crane ('21) found to be the highest H-ion concentration in which *Paramecium* can live 24 hours. I have checked this observation and have obtained essentially the same results.

Close observations demonstrate rather clearly that *Paramecia* give the same response upon coming into contact with strong acids (more acid than pH 5.0) and upon coming into contact with their old culture medium after they are already in the acid solution (less acid than pH 5.0). In both cases they give the avoiding reaction.

Dr. L. G. Barth in his Doctor's Dissertation,[1] "The Effect of Acids and Alkalies on the Viscosity of Protoplasm," has found in unfertilized *Arbacia* eggs that acids if they are able to penetrate coagulate at pH 5.0. This suggests a reason why *Paramecia* are not able to live in concentrations of acids lower than pH 5.0.

Incidentally some observations were made on the toxicity of acid solutions. Pruthi ('27) observed that animals from a culture with a concentration of pH 7.8 placed in a hydrochloric acid solution with a concentration of pH 6.0 died within half an hour. Throughout these experiments it was observed that the organisms were in good condition after being in even stronger acids for considerable periods. Some *Paramecia* from a culture with a concentration of pH 7.8 were stirred into a citric acid solution with a concentration of pH 5.7. At the end of a half hour and an hour and a half some were placed back in culture media. In each case the animals seemed to suffer no ill effects and reproduced in an apparently normal manner. Again some *Paramecia* were placed in a hydrochloric acid solution with a concentration of pH 6.0 and left. Three days later the H-ion concentration had fallen to pH 6.2 and the animals were in normal condition. Pruthi also observed that *Paramecia* reproduce more rapidly in their own culture medium than in tap-water of the same H-ion concentration and suggests that this may be due to a greater amount of carbonates in the culture medium. To the writer of this paper it seems that a much more definite factor is food, as rate of division is closely correlated to amounts of food available.

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[1] Placed in the University of Chicago Library in 1929. To be published later.

Jennings' ('06) statement that "*Paramecia* collect in all weakly acid solutions, no matter what acid substance is present," was confirmed in these experiments. In the cases of pyrogallic acid and tannic acid no positive responses were obtained at the H-ion concentration used. To make solutions of these two acids with a pH range of 4.8–5.8, almost saturated solutions were necessary. It seems that some other factor besides the H-ion concentration is responsible for the great toxicity of these two acids. However, it was interesting to note that *Paramecia* reacted to weaker solutions of these two acids. In each case when pyrogallic or tannic acid was used the *Paramecia* gathered in the neck between the two drops and died there. This bears out another statement by Jennings to the effect that there is no relationship between the attracting or repelling power of acids and their injurious effects. Since it was apparent that all acids elicited the same responses, unless they were toxic in the pH range used, the three inorganic acids, hydrochloric, sulphuric and nitric acids, were used in most of the experiments in order that a more concentrated study might be made.

It is well known that carbon dioxide plays an important part in the behavior of *Paramecia* in natural conditions. The work of Hyman on Planaria ('25) made it appear that *Paramecia*, in reacting to acidified water, might be reacting to the carbon dioxide liberated from the carbonates instead of to free hydrogen ions. The series of experiments using carbonate-free water were run with the aim of finding out something in this connection. Although the writer realizes that many biologists today feel that too much significance has been ascribed to H-ion concentration "per se" in biological reactions, the results of the experiments using carbonate-free water give a strong indication that *Paramecia*, when reacting to weakly acid solutions, react to the free hydrogen ions. This idea does not seem to be out of line with the natural conditions that cause *Paramecia* to gather in clumps where carbon dioxide is present. The carbon dioxide in the cultures where *Paramecia* gather in clumps is in the form of carbonic acid.

Jennings ('06) has noted that all alkalies, save the alums, have a strong repellent effect on *Paramecia*. The observations made in these experiments that *Paramecia* will not react positively to a H-ion concentration lower than that of their culture tend to support this conclusion. The repellent effect of alkalies on *Paramecia* seems to help explain the fact that *Paramecia*, in cultures which have become quite alkaline (so far as is known the change in H-ion concentration in cultures cannot be regulated by the *Paramecia*), react to solutions with a H-ion concentration higher than that of their cultures.

Loeb ('18) based his conclusion that *Paramecia* are indifferent to acid solutions on Barratt's experiments using Pfeffer's capillary tubes. In repeating Barratt's work it seemed evident that the size of the openings in the tubes is the factor which explains the indifferent reactions. *Paramecia* cannot enter the small tubes without striking the edges. They can and will enter larger tubes containing weakly acid solutions.

The nature of the response of *Paramecia* to acid solutions received some consideration also. Jennings ('99) believed that the aggregations resulted from trap action, *i.e.* from a series of negative reactions to solutions of low acidity when the animals were in acid, rather than from a true positive chemotropism. Garrey ('02) working with *Chilomonas* concluded that the organisms showed positive chemotropism to acetic, lactic, and butyric acids, but that aggregations in inorganic acids resulted from trap action or "chemokinesis" as he called it. His conclusion was made possible by the manner of orientation and slow movement of the organisms he studied. The rapid movement of *Paramecia* through diffusion areas prevents accurate conclusions as to the nature of the responses to acids, so it was not possible to distinguish between the two types of reaction as observed by Garrey. It seemed evident, in these experiments, that the *Paramecia* reached the acid region by trial or random movements, and that once within the acid trap action occurred. Crossing back and forth between drops which had stood a long time was often noticed but in such cases the indicators would usually show that complete diffusion had taken place throughout both drops.

In the experiments when carbonate-free water or distilled water was used NaOH was added to lower the H-ion concentration in each case to pH 7.8. In all of these experiments the *Paramecia* tended to gather in clumps in a much more striking way than they did in any of the other experiments. In some cases the clumping occurred in the neck between the two drops, making it necessary to discard such experiments. In these experiments hydrochloric, sulphuric, nitric and carbonic acids were added to the water, altered with NaOH to produce the desired H-ion concentrations. As a result NaCl, Na_2SO_4, $NaNO_3$ or Na_2CO_3 was formed in the solutions. Jennings ('06) has listed NaCl, $NaNO_3$ and Na_2CO_3 as being very repellent to *Paramecia*. He further states that when a repellent substance, as NaCl, is mixed with an acid and a drop of the mixture is placed on a slide of *Paramecia* the *Paramecia* gather in a ring about the drop. In these experiments it seems that the attracting power of the H-ions is greater than the repellent power of the salts formed.

One of the most puzzling features of the whole series of experiments was the failure of the *Paramecia* to react positively to Whitman well water. Although analyses of Whitman well water and Hull well water were obtained for reference, no possible cause for the difference in eliciting positive responses can be given at this time. The *Paramecia* seem to serve as very delicate indicators in this connection, and the fact that *Paramecia* do not react positively to Whitman well water may be of importance in future experimentation with this water.

SUMMARY.

1. The reactions of *Paramecium caudatum* to solutions of known hydrogen ion concentration were studied.
2. The colorimetric method of determining hydrogen ion

concentration was used but was checked by a series determined both colorimetrically and by Youden's potentiometer.

3. The acids used were: hydrochloric, nitric, sulphuric, carbonic, formic, acetic, citric, picric, pyrogallic and tannic.

4. The waters used were: Chicago tap-water, Whitman well water, Hull well water, Crawfordsville (Ind.) tap-water, distilled water, Chicago tap-water made carbonate-free, Whitman well water made carbonate-free, and Hull well water made carbonate-free.

5. In these experiments only *Paramecia* from alkaline cultures responded positively to acidified solutions.

6. *Paramecia* from alkaline cultures react positively to all H-ion concentrations higher than that of their culture to pH 5.0 (4.83 potentiometer reading) inclusive, when the H-ion concentration is produced by the inorganic acids—hydrochloric, sulphuric and nitric acids.

7. Organic acids, such as acetic, formic, carbonic and citric, which are not toxic in this pH range elicit responses similar to those caused by the inorganic acids.

8. Pyrogallic and tannic acids are toxic to *Paramecia* in solutions with concentrations of pH 4.8 through pH 5.8 but the organisms enter these waters and die as a result.

9. *Paramecia* react to acidified water from which all carbonates have been previously removed. This is an indication that *Paramecia* react to the hydrogen ion "per se" and not to carbon dioxide.

10. *Paramecia* are better able to live in solutions of high H-ion concentration than many have supposed.

11. Sulphonphthalein indicators, when used in no stronger concentration than necessary for pH determinations, are not harmful to *Paramecia*.

12. *Paramecia* will not react positively to weakly acid solutions when the temperature is below 21 degrees C.

13. No new evidence was obtained as to the nature of the mechanics of the reaction of *Paramecia* to acids.

14. *Paramecia* gather in clumps when they react to acidified solutions containing NaOH.

15. In one of the four types of water tested acidified water did

not elicit positive responses from *Paramecia*. No reason is offered for this exception.

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BIBLIOGRAPHY.

Beers, C. F.

'27 The Relation between Hydrogen Ion Concentration and Encystment in *Didinium nasutum*. Jour. Morph. and Physiol., XLIV., 21–28.

Bodine, J. H.

'21 Hydrogen Ion Concentration of Protozoan Cultures. Biol. Bull., XLI., 73–78.

Clark, W. M., and Lubs, H. A.

'27 The Colorimetric Determination of Hydrogen Ion Concentration. Jour. Bact., II., 109–236.

Child, C. M., and Deviney, Ezda.

'26 Physiology of *Paramecium*. Jour. Exp. Zoöl., XLIII., 257–312.

Crane, Marian M.

'21 Toxicity of Alkaloids for *Paramecium*. Jour. Pharm. and Exp. Therapeutics, XVIII., 319–329.

Collett, M. E.

'19 Toxicity of Acids to Ciliate Infusoria. Jour. Exp. Zoöl., XXIX., 443–473.

Darby, H. H.

'29 The Effect of the H-ion Concentration. Archiv für Protistenkunde, LXV., 1–35.

Eddy, Samuel.

'28 Succession of Protozoa in Cultures under Controlled Conditions. Transactions of Am. Micros. Soc., XLVII., 283–304.

Fine, M. S.

'12 Chemical Properties of Hay Infusions with Reference to Acidity and Its Relation to Protozoan Sequence. Jour. Exp. Zoöl., XII., 265–282.

Garrey, W. E.

'00 Effect of Ions on Aggregations of Flagellated Infusoria. Am. Jour. Physiol., III., 291–315.

Greeley, A. W.

'04 Structure of Protoplasm of *Paramecium*. Biol. Bull., VII., 1–32.

Hopkins, D. L.

'26 The Effect of Hydrogen Ion Concentration on Locomotion and Other Life Processes in *Amœba proteus*. Proc. N. A. S., XII., 311–315.

Hyman, L. H.

'25 Oxygen Consumption in Acids. Biol. Bull., XL., 288–332.

Jennings, H. S.

'97 Reactions to Chemical, Osmotic, and Mechanical Stimuli in the Ciliate Infusoria. Jour. Physiol., XXI., 258–322.

'99 The Mechanism of the Motor Reactions of *Paramecium*. Am. Jour. Physiol., II., 311–341.

'99 Reactions to Localized Stimuli in *Spirostomum* and *Stentor*. Am. Nat., XXXIII., 373–389.

'99 Laws of Chemotaxis in *Paramecium*. Am. Jour. Physiol., II., 355–379.

'00 On the Movements and Motor Reflexes of the Flagellata and Ciliata. Am. Jour. Physiol., III., 229–260.

Jennings, H. S.

'00 On the Reactions of *Chilomonas* to Organic Acids. Am. Jour. Physiol., III., 397–403.

'00 Reactions of Infusoria to Chemicals: a Criticism. Am. Nat., XXXIV., 259–265.

Jennings, H. S., and Moore, E. H.

'02 On the Reactions of Infusoria to Carbonic Acids with Especial Reference to the Causes of the Gatherings Spontaneously Formed. Am. Jour. Physiol., VI., 233–250.

Jennings, H. S.

'06 Behavior of the Lower Organisms. New York (Columbia Univ. Press), 59–109.

Kostir, W. J.

'21 Comparative Resistance of Different Species of *Euglenidæ* to Citric Acid. Ohio Jour. Sci., XXI., 267–271.

Loeb, Jacques.

'18 Forced Movements, Tropisms, and Animal Conduct. Philadelphia (Lippincott), 139–154.

Pruthi, Hem Singh.

'27 On the Hydrogen Ion Concentration of Hay Infusions with Specific Reference to Its Influence in Protozoan Sequence. Br. Jour. of Exp. Biol., IV., 292–300.

Saunders, J. T.

'24 Effect of Hydrogen Ion Concentration on Behavior, Growth, and Occurrence of *Spirostomum*. Proc. Camb. Phil. Soc. (Biol.), 189–203.

Wenrich, D. H.

'28 Eight Well-defined Species of *Paramecium*. Transactions of Am. Micros. Soc., XLVII., 275–282.

REGENERATION IN *POLYCHŒRUS CAUDATUS* MARK.[1]

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INTRODUCTION.

The purpose of these observations was to re-examine and supplement the investigations of Stevens (1905), and Child (1907) on the regeneration in the accœlous flat worm—*Polychœrus caudatus.*

The observations and experiments on living material were carried out at the Marine Biological Laboratory, Woods Hole, during the summers of 1927, 1928, 1929.

HISTORICAL

Polychœrus caudatus was given its name by Mark in a paper which contains an excellent description of the external and internal anatomy (1892). Gardiner published papers on, "The early development of *Polychœrus*" (1895), and "The growth of the ovum, formation of the polar bodies, and the fertilization in *Polychœrus caudatus*" (1898). Löhner in 1910 examined Mark's description and added a more detailed account of the nervous system.

Regeneration in *Polychœrus* was first described by Stevens and Boring (1905)—Miss Stevens doing the experiments on the living material and Miss Boring the histology of regeneration. Miss Stevens sectioned the worms by two general type cuts, that is, by transverse cuts at two levels of the body, and also by longitudinal oblique cuts. She observed regeneration in almost all these pieces, the only exception being a failure of anterior regeneration in some of the "middle pieces." Miss Boring concluded that regeneration in *Polychœrus* was by morphallaxis.

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[1] I wish to express my sincere appreciation to Professor J. Walter Wilson whose helpful suggestions made these investigations possible; and to Dr. William A. Castle who also read the manuscipt.

Child (1907) gave a more detailed and extensive account of regeneration. Using the statocyst as a landmark in locating the cephalic ganglion, he made transverse, longitudinal and oblique cuts. In the transverse cuts he noted that "posterior regulation does not occur in the preganglionic region." As the transverse cuts were made progressing more posteriorly toward the caudal lobes, the amount of posterior regeneration gradually decreased and redifferentiation increased. Anterior regulation in pieces anterior to the ganglia was mostly by redifferentiation, while directly posterior to the ganglia it was by regeneration. As the level of cut proceeded toward the caudal lobes, anterior regeneration gradually decreased until at levels in the posterior fourth of the animal, it was limited to wound closure. In observing lateral regulation, Child found that the 'half' animal without the statocyst never regenerated its lost half, while the 'half' containing the statocyst showed complete regeneration. Oblique regulation proved to be a combination of lateral and longitudinal regulation. Child considered these facts to show that a relation existed between the nervous system and "form regulation" which appeared to be functional.

Material and Methods.

About six hundred specimens were used in this study of regeneration. They were collected in Little Harbor at Woods Hole, where at low tide great numbers are easily found on the green Ulva.

In the laboratory they were kept in large shallow dishes in a cool shaded place, the sea water being changed every other day. No attempt was made to feed the worms, and therefore they decreased gradually in size through lack of food.

The individual animals were isolated by means of wide-mouthed pipettes. To operate, the worms were transferred into a Syracuse watch glass which had been filled with paraffin. All the operations were performed with a sharp scalpel under the binocular microscope. The different series of operated animals were then placed in labeled Stender dishes, and daily observations were made.

In order to record and measure the cuts with respect to the position of the statocyst, it was necessary to isolate the operated animal upon a glass slide (under low power) and gently press the cover slip. The pressure was controlled by vaseline, and only in this way could the writer see the statocyst. It appears somewhat like a small air bubble and can be easily missed.

Figures were drawn from the living experimental animals as accurately as possible.

EXPERIMENTAL OBSERVATIONS.

In the following discussion of experimental observations, the author is using Morgan's terminology for the restoration of parts. "Morphallaxis" is interpreted as the remolding of old tissue directly into a new form without a proliferation at the cut surface. Parts are replaced by "epimorphosis" when there is a "proliferation of material preceding the development of the new part" (1901, p. 23), that is, an outgrowth of new tissue from which the missing part may develop. Morgan includes both of these processes under the single word "regeneration" which he uses to signify the restoration of form. Child uses the term "regulation" as parallel to Morgan's "regeneration." This "regulation" may occur either by "redifferentiation" of old tissue (Morgan's morphallaxis) or by "regeneration" (Morgan's epimorphosis).

In a number of the following experiments, the type of cut has been made in relation to the position of the main ganglionic masses. A short account of the nervous system, therefore, seems necessary.

Mark's anatomical description of the nervous system shows "a pair of ganglionic masses connected by a transverse commisure which lies above the otocyst" (p. 304). From these ganglionic masses nerve trunks arise. Mark considers the chief nerves of this type of system rather comparable to that described in *Convoluta* by Délage (1886) and von Graff (1891).

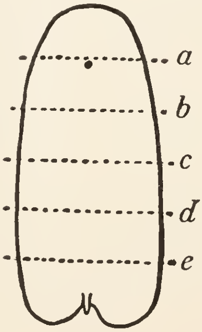
In 1910 Löhner, a student of von Graff, made an extensive histological study of *Polychœrus*, including a detailed account of the nervous system. He found that *Polychœrus* had one median unpaired ganglion, and two lateral ganglia symmetrically placed —that is, one on either side of the median ganglion, approximately mid-way between the median ganglion and the lateral edge of the animal. The statocyst he found to be imbedded in the median ganglion (see Löhner's diagram on Plate 17, or Hanström, p. 91).

In this paper the author is following Löhner's description of the nervous system and therefore, the use of the words "main ganglionic masses" refers to the three ganglia as described above.

The first experiments were to re-examine the type cuts made by Child, and to supplement with slight modification.

Posterior and Anterior Regeneration.

Transverse sections were made at five different levels as indicated in Fig. 1, and regeneration at each level was observed. Posterior and anterior regeneration was noticed to agree with Child's observations (1907) at comparable levels. That is, if a worm is cut in front of the ganglia, the anterior piece neither regenerates new caudal lobes by epimorphosis nor reorganizes to a normal form.



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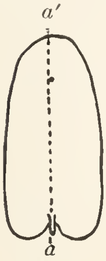
If a worm is cut posterior to the ganglia at levels preceding toward the caudal lobes—the amount of posterior epimorphosis decreases and reorganization increases as the level of cut reaches the posterior end.

The posterior piece of a worm that had been sectioned anterior to the ganglia, shows complete regeneration by morphallaxis rather than by epimorphosis. At all levels posterior to the ganglia, anterior regeneration was never complete—the amount of epimorphosis decreasing as the cuts preceded towards the caudal lobes.

Lateral Regeneration.

It was in lateral regeneration that a difference was noted between Child's experiments (1907) and those recorded in this paper.

Sections were made along *a a′* (Fig. 2) and the amounts and rates of regeneration in the two halves were compared. Immediately after the operation, the worm contracted on the side toward the cut surface, thereafter moving in a circle. Ten days after sectioning the pieces without the statocyst appeared to have regenerated just as much as did the pieces containing the statocyst (Fig. 3, 4) and movement was not in a circular direction. After twenty days, the "half" without the statocyst and median ganglionic mass, had formed a distinct caudal lobe, and the amount of new tissue was almost equal to that in the other "half" (Fig. 5, 6). It is surprising to note that the "half" without the statocyst behaved at first as a "headless piece," but as might be expected, when regenerated tissue appeared, the behavior of the two "halves" did not differ when mechanically stimulated.



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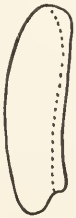

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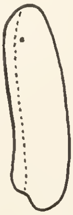

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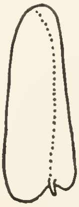

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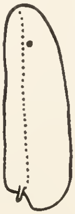

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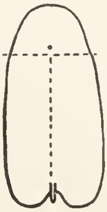

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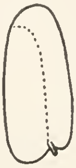

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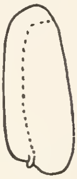

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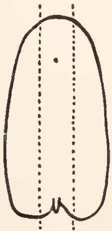

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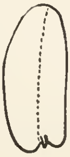

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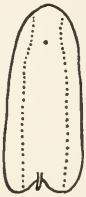

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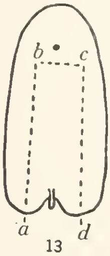
A combination of lateral and anterior regeneration was then made (Fig. 7), to compare with the above lateral regeneration. Seven days after the operation, all the pieces regenerated both anteriorly and laterally (Fig. 8, 9).

The results of sectioning as indicated in Fig. 10, dividing the animal into three parts, showed regeneration in the following manner. Within six days each marginal piece had formed by epimorphosis a new caudal lobe, while the old tissue at the posterior margin extended posteriorly and reorganized, thus replacing the other lobe (Fig. 11). As might be expected, no statocyst could be identified in either marginal piece, although the animals were more active than the so-called 'headless piece' produced by anterior cuts. The middle pieces, containing the statocysts, did not regenerate as rapidly as did the marginal pieces—the anterior part of the animal showing the more rapid lateral regeneration. Within two weeks, however, the middle pieces, by epimorphosis and morphallaxis appeared almost normal (Fig. 12).

Some modifications of lateral cuts were made as shown in Fig. 13. The animals were cut along line *a b c d*, leaving the statocyst in place. Regeneration was very rapid, taking place within five days. Each marginal piece regenerated laterally, and formed a caudal lobe with filaments as in Fig. 14. The animal had the appearance of two animals with one head, but moved as one. In one instance, the 'one animal' did not develop any caudal lobes, thus giving an asymmetrical form (Fig. 15).

Sections were then made (Fig. 16) to find if there were lateral regeneration in the absence of the median ganglionic mass. Apparently there was no obvious correlation, for regeneration was neither retarded nor modified. If "they" had not pulled apart, then the *Polychœrus* appeared as in Fig. 17, five days after the operation. However, sections cut as in Fig. 18, showed slight variations. Due to the fact that these worms were less active, the greater part of the lateral cut surfaces healed, and the animal appeared as in Fig. 19. The fate of that portion of the animal indicated by cross hatching in Fig. 16, was also observed. These pieces did not regenerate beyond wound closure either laterally or anteriorly, and as a result of their inability to regenerate, the animals often showed a partial union of the cut edges at the anterior portion. The caudal tissue maintained its function of attachment (Fig. 20).



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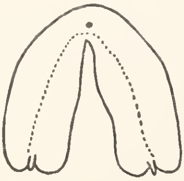

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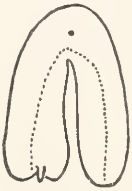

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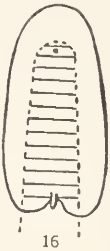

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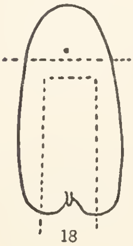

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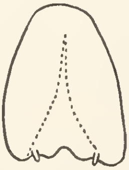

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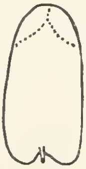

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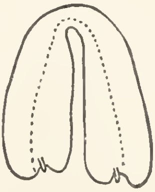

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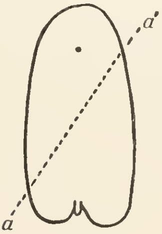
Oblique Regeneration.

Animals were sectioned along *a a′* (Fig. 21). The results were as might be expected from combinations of lateral and longitudinal regeneration. The posterior marginal edge reorganized into a right caudal lobe, while the left one was formed by epimorphosis (Fig. 22).



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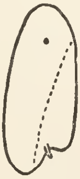

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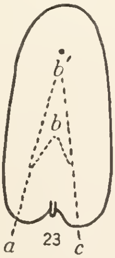

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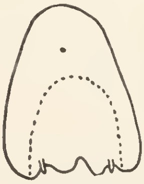

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Some combinations of oblique cuts gave interesting results. If the animals were sectioned along *a b c* (Fig. 23), the anterior part of the animal regenerated two pairs of caudal lobes with tail filaments (Fig. 24).

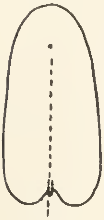
Similar results are observed later as in Fig. 26. The more anterior the cut, the greater the amount of lateral replacement. If the section removed extended to just behind the statocyst (*a b′ c*, Fig. 23), the amount of lateral replacement was so great as to give the appearance of a form shown by Fig. 14.

Regeneration Following Other Types of Cuts.

Longitudinal incisions were made through the caudal lobes and extending anteriorly to the statocyst—thus dividing the animal posteriorly into two parts (Fig. 25). *Polychœrus* showed remarkable powers of healing. Within an hour the animal healed almost the entire length of the cut, a small portion of the caudal part of the incision remaining open. The result of this cut was that two or three pairs of caudal lobes often appeared where one pair was normally present. Tail filaments appeared at the caudal notches (Fig. 26).

The same type of incision was made at the anterior end. The animals healed within an hour and behaved normally. No obvious morphallaxis nor epimorphosis was noted in these anterior cuts.

When large circular pieces from the center of the worm were removed leaving a "living ring" (Fig. 27), new tissue rapidly regenerated from the cut surface, and replaced the tissue removed. If the wound did not close within four days, the regenerated tissue never closed the opening. In such cases, an open slit was left until death (Fig. 28). If the circular piece was not quite as large as in Fig. 27, the animal again displayed its remarkable healing and reorganizing powers. The wounded surface was drawn together, and within seven to ten days by morphallaxis and epimorphosis, the animal assumed its normal appearance. Fig. 29–30 show two unusual results from animals that had been cut as is indicated in Fig. 27. The "living ring" was broken at the region of the caudal notch (shown by dotted lines in Fig. 27), and by nine days the lateral regeneration of two pairs of caudal lobes with filaments, had taken place. A week later, a new growth of tissue appeared at the notch, which extended and pulled posteriorly as a head might at such a location. The animal lived about a month after the operation, and up to that time, no statocyst had developed in this regenerated 'head.' These observations recall rather similar results in *Planaria* observed by Lemon (1899), Van Duyne (1896), Bardeen (1901) and Morgan (1900) which show the formation of a head in the notch of the cut. Many animals were cut similarly in hopes of obtaining more of the same results, but to the time of writing, no more have occurred.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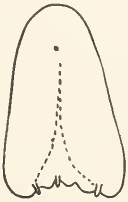
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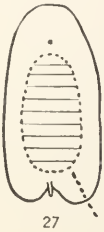
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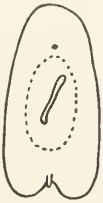
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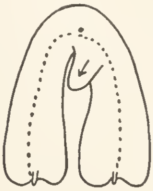
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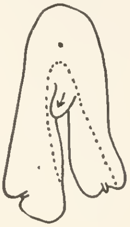
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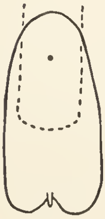
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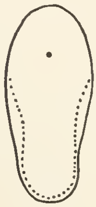
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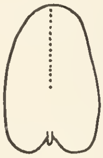
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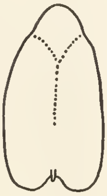
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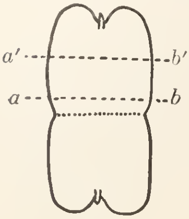
If the animals were cut as shown in Fig. 31, the anterior portion formed normal lobes and filaments by lateral and posterior regeneration. In one instance (Fig. 32) there was regeneration of the missing lateral part, but no posterior regeneration beyond wound closure. The posterior part of the cut worm was also observed. Within an hour the worm showed wound closure by drawing the cut surfaces together (Fig. 33). Ten days later, regenerated tissue began to push out gradually (Fig. 34). About two weeks later the animal assumed its normal form again, although no new statocyst was ever observed. In one or two instances regeneration did not occur. The worm remained as Fig. 33 and showed marked thickening until death.

EXPERIMENTAL GRAFTING.

The rapid healing ability noted in *Polychœrus*, suggested the possibility of successful grafts and the regeneration of grafted pieces.

The object of these grafting experiments was to study the possibility of reversal of polarity in *Polychœrus*.

Lillian Morgan's technique used for uniting desired pieces of *Planarians* (*Planaria maculata* and *Phagocata gracilis*) was tried unsuccessfully on *Polychœrus*. It appeared that *Polychœrus* needed more water than was offered by moist filter paper. The 'chance method' of union, that is, cutting the worms in two, and placing together about forty of the posterior pieces in a salt cellar, gave the best results. In about 16 per cent. grafts were obtained in which two pieces were united by their anterior cut surfaces (Fig. 35). These grafted pieces were then isolated into separate dishes. The union was quite perfect, the region of union almost indistinguishable and usually no regeneration occurred. The compound animal, however, always behaved as two separate individuals, and slight locomotion was possible



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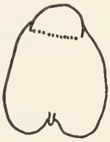

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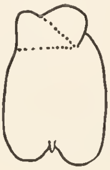

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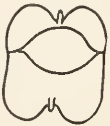

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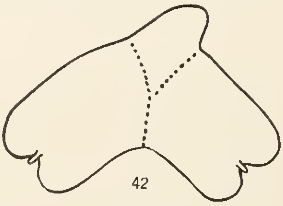

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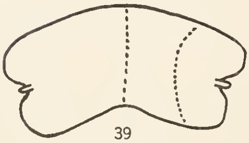

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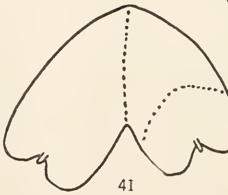

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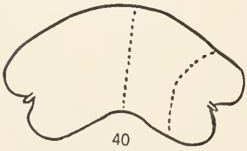

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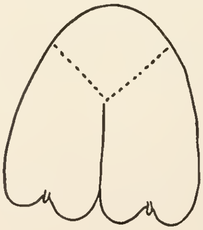

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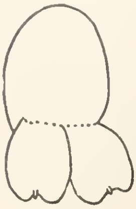
only when one individual lost its hold upon the substrate. It then might be pushed about by the other piece.

Ten grafts were cut in two, at a level very near the region of union of the two pieces (lines *a b*, Fig. 35). Eight of these, neither regenerated beyond wound closure at the cut surface, nor regenerated at the region of union. The smaller grafted pieces rounded up, showing marked dorsal thickening (Fig. 36) The pieces were watched for twenty days. Of the remaining two, one piece regenerated at the region of union within three days after the cut. The "rounded up" tissue appeared to be pushed to the side, as the larger piece regenerated its anterior tissue (Fig. 37). The other piece showed regeneration of caudal lobes and a tail filament at the cut surface (Fig. 38).

In another experiment, twelve grafted compound animals were cut at a different level (*a′ b′*, Fig. 35), farther away from the region of union. In every instance caudal lobes and filaments were formed. A slight locomotion was gradually accomplished by the worms assuming positions as shown by Fig. 39, 40, 41. In three instances the compound animals not only regenerated the lost lobes, but also "heads" at the region of union (in this "head" no statocyst could be identified). This "head" did not seem to belong especially to either component part, but pushed out from between the pieces (Fig. 42, 43). These experiments with reverse grafted pieces recall Lillian Morgan's (1906) experiments with reverse grafted pieces of *Planaria* in which a head formed from the line of union; and Joest's experiments with *Allolobophora terrestris*, when he grafted together the anterior ends of two worms, and one or two heads developed at the region of union.

In some of these grafts, before the second cut was made on the compound animal, the dorsal surface of the one animal was continuous with the ventral surface of the other (one animal was on its back when the graft formed). It was found that either the caudal lobes of one component were used for attachment; or both caudal lobes attached themselves to the substrate by their ventral surfaces. In the later instance, the animal was twisted at the region of union, the twist assuming the form of a bulge.

Two other combinations of pieces were formed by the "chance method." Fig. 44 shows two small posterior pieces attached to the posterior portion of a larger anterior piece. In Fig. 45, a posterior part is shown attached to an anterior piece, the dorsal surface of one animal continuous with the ventral surface of the other. They remained in this form until death.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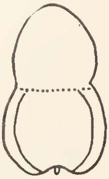
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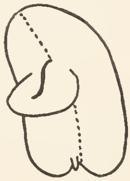
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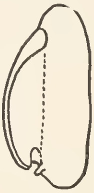
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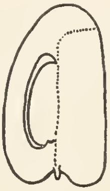
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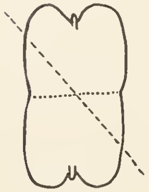
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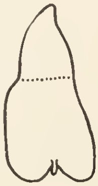
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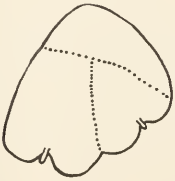
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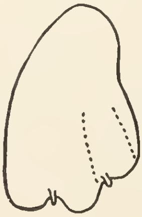
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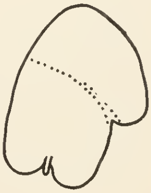
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In one instance a graft occurred in a cut like that described in Fig. 10. The union between the middle and right marginal piece, with a different orientation, resulted in a form as shown in Fig. 46. The larger piece regenerated laterally while the smaller grafted piece seemed to be gradually absorbed by the larger.

Another example of absorption of the smaller grafted piece is shown by Fig. 47, 48. This graft occurred between the two lateral pieces of a worm cut as shown in Fig. 7. The graft was such that ventral and dorsal surfaces were continuous. The larger piece regenerated laterally and anteriorly and therefore pushed the smaller graft on its dorsal side. A gradual absorption of the smaller piece occurred.

In another series of graft experiments, the anterior ends of the animals were united, and later the pieces were cut obliquely through the line of union (Fig. 49). Twenty-four hours after, the small grafted piece still functioned as a caudal piece, although the cut surfaces contracted thus giving the appearance of Fig. 50. It was difficult to distinguish the union of the graft in the contracted worm. Ten days later, new caudal lobes and a filament had developed in a number of animals, and the worms assumed the relative position shown by Fig. 51. Whether the caudal lobes developed entirely from the small graft or from the larger piece, could not be detected (Fig. 51, 52). (A difficulty of the same sort is mentioned by Morgan in similar cuts on grafted *Bipalium kewense.*)

In a few cases the cut oblique portions healed together and no regeneration occurred beyond wound closure, thus giving the appearance as in Fig. 53.

A series of oblique regeneration from grafted pieces was made, in which one piece of the graft was vitally stained with Nile Blue Sulphate. It was hoped that in this way the exact origin of new tissue (Fig. 51–52) could be determined. In this respect the results of this experiment were unsuccessful for two reasons—*i.e.*, the stain had to be of such a dilute nature in order to be vital, that the detection of the dye in the regenerated portion was not apparent, or the stained portion of the graft was sluffed off before regeneration could occur.

Attempts were made to graft two anterior ends by their posterior cut surfaces. The pieces, however, crawled away from each other so that all attempts of this kind proved unsuccessful.

DISCUSSION.

From Child's experiments (1907) and the authors' observations it would seem evident that the absence of the median and lateral ganglion prevent complete anterior and posterior regeneration.

The difference in rate and method of locomotion between animals with and without the ganglia was noticeable for some time after the operation. This time may be from five to ten days. The 'headless' pieces at first appeared more sluggish than the others, but within two weeks they behaved quite normally—provided that dorsal thickening had not taken place.

It appears that lateral regeneration occurs in the absence of the median ganglionic mass (Fig. 3, 4, 5, 7). This seems to agree with L. V. Morgan's observations in *Leptoplana littoralis* (1905, p. 193). Since it is thought that the lateral ganglion and likewise the nerve roots near the median ganglion still remain in *Polychœrus* when sectioned as in Fig. 3 (as Child suggested with reference to *Leptoplana littoralis*—1910, p. 338), it might seem that their presence might influence lateral regeneration. But in comparing modifications of lateral cuts (Fig. 16, 18) it again indicates that in *Polychœrus*, the median and lateral ganglia themselves do not play such an essential rôle in regeneration as would be expected. In the experiments reported in this paper, the worms regenerated always into a normal form, although regeneration was retarded slightly and the statocyst was never obviously replaced.

In recent correspondence with Dr. Child concerning this apparent disagreement between his observations and mine, that is, regeneration from pieces of *Polychœrus* cut in the median lateral plane without the median ganglion (Child's Fig. 31), Dr. Child offers several possible interpretations:

First, a species difference. The species of *Polychœrus* studied at Pacific Grove, California, and that at Woods Hole, Massachusetts, may not be the same and ". . . differ slightly as regards the ganglionic distribution."

Second. The longitudinal cuts described by Child may be somewhat more lateral than those described in this paper, and therefore the amount of the ganglionic masses would be different.

Third. The pieces of *Polychœrus* in lateral regeneration as described by Child were not observed as long as other pieces. Dr. Child writes, "I believe that these particular pieces were not followed as long as some others and that they might perhaps later have shown a change and come to resemble more or less closely those which you describe."

Fourth. ". . . the possibility that differences in physiological age and development might account for differences of this sort."

With respect to these differences Dr. Child remarks, "in any case the difference seems to me of minor importance and to indicate that some difference in material or experimental conditions had been involved in the two cases."

As the longitudinal cuts approached the margin of the animal, epimorphosis decreased and morphallaxis increased. This confirms Child's theory of functional substitution, as stated in the historical summary of this paper.

Whenever the marginal posterior part of the animal is separated from its other margin, two or more caudal lobes are formed (Fig. 14, 15, 17, 24, 29, 30). This recalls the work of T. H. Morgan (1900) and of Randolph (1897), who produced double tails in *Planaria* by oblique cuts.

By reviewing the cuts which gave rise to the two tailed worms (Fig. 13, 16, 18, 23, 25) it appears that the length of the regenerated posterior region may be controlled by the depth or length of the cut and by the amount of wound closure. Note in Fig. 14 and 17 that the cuts extended far anteriorly and separated the marginal parts of the animals. These cuts gave rise to two distinct posterior parts, that is, four caudal lobes with a filament in the two notches. Cuts which did not extend so far anteriorly and showed more wound closure, resulted in a smaller amount of posterior regeneration (Fig. 19, 24, 26).

Miss Stevens observed in the "posterior regulation" of a few "middle pieces" a regeneration of supernumerary appendages and caudal lobes. In repeating these experiments similar results have been obtained only when the cut separated the posterior region. (The writer is lead to think that the supernumerary appendages described by Miss Stevens in her Fig. N, were due to a slight wound in these middle pieces caused when they were "violently disturbed," p. 339.) Miss Stevens referring to these extra appendages remarks that, "all the variations observed in regeneration are to be found in normal adult worms" (p. 337). Such a statement appears startling, for in the hundreds of normal worms observed, the writer has seen only three or four individuals that showed more than the normal number of lobes and appendages, but by close observation it was seen that a wound had formed and regeneration had taken place before the worms had been brought into the laboratory, thus giving the animal three or four lobes with four or five filaments. Mark in his description of the species says, "I have never seen an individual with more than three tail filaments" (p. 301), nor does Child mention such extra normal tail filaments in his account of regeneration.

The rapid wound closure taking place in animals that had been cut by a circular incision (Fig. 27), agrees with Child's remark that in *Turbellaria* and in many other forms "nutritive and other conditions are better in such a cleft, where the growing parts are in contact on both sides with other tissue, than on surfaces where such contact exists only on one side" (1910, p. 334). When the cuts were circular (Fig. 27), if neither the cut surfaces of the worm fused together nor the regenerated tissue closed the opening within four or five days, then the slit remained open as long as observations was continued (Fig. 28). It appears that the muscles about the open slit, do not continue to contract after new tissue had been formed for three or four days. The muscles then relax as in a normal regeneration of tissue.

Instances similar to the two where head formation occurred in the angles between the posterior parts (Fig. 29, 30), were never observed again. Cuts indicated by Fig. 13, 23, 25, 27 were expected to give similar results. (When the cut surfaces healed immediately, head formation was not expected.) Van Duyne, Lemon, Bardeen and Morgan easily obtained similar heads in *Planaria* by making longitudinal incisions in the tail, forward to about the level of the eye spots. Such a head formation in *Polychœrus* should not be mistaken for a true heteromorphic head. Morgan unlike Van Duyne, does not interpret such heads in *Planaria* as heteromorphic heads—"the new heads develop in connection with the new material of the half, and appear at the side as does the new head in longitudinal pieces, and are, therefore, not, in one sense, heteromorphic productions" (1900, p. 98).

In grafting pieces of *Polychœrus*, so that the posterior ends were united by their anterior cut surfaces, it was noted that when grafts were cut at a level near to the region of union, in most cases the smaller grafted pieces failed to regenerate caudal obes, and rounded up by extreme dorsal thickening. In the other type cut, in which the cut was farther from the region of union, caudal lobes and filaments always regenerated. Therefore, in both these types of regeneration from grafted pieces, no reversal of polarity occurred. This seems to agree with L. Morgan's experiment with pieces from the middle region of *Planaria* which had been reversed and grafted to the head region of another.

Child suggests that in *Polychœrus* there exists a relation between the central nervous system and form regulation. In my experiments the prevention or delay of regeneration in the one type of grafted cut (Fig. 35 *a b*), may then be due to the fact that the grafted piece is very small and therefore contains a smaller amount of the longitudinal nerve trunks than it does in the other type of graft cut (Fig. 35 *a′ b′*).

Since grafts did not occur between extreme anterior ends of *Polychœrus*, the regeneration of reversed pieces from the head region could not be watched. Whether the polarity of the longer piece might have influenced the smaller grafted piece (Fig. 36, 37, 51, 52, 53), as it appeared to do in L. Morgan's experiment with *Planaria*, still remains to be shown.

The appearance of a head in some instances as the region of union (Fig. 37, 42) and its lack of appearance at other times, might indicate that the cut surfaces of the graft were not united their entire length. This would occur if the worms were of a different size. In the case shown by Fig. 37, the regenerated head seemed to originate from the larger piece, and it assumed the position of the larger pieces' head. However, in the case shown by Fig. 42, the head seemed not to belong specifically to either graft. The head continued to grow larger and morphallaxis seemed to occur in both the grafts—the head becoming as broad as both component grafts (Fig. 43).

Summary and Conclusions.

1. If a worm is cut in front of the ganglia, the anterior piece neither regenerates new caudal lobes by epimorphosis nor reorganizes to a normal form.

2. If a worm is cut posterior to the ganglia at levels preceding toward the caudal lobes—the amount of posterior epimorphosis decreases and reorganization increases as the level of cut reaches the posterior end.

3. The posterior piece of a worm that had been sectioned

anterior to the ganglia, shows complete regeneration by morphallaxis rather than by epimorphosis. At all levels posterior to the ganglia, anterior regeneration was never complete—the amount of epimorphosis decreasing as the cuts proceeded towards the caudal lobes.

4. If a worm is cut longitudinally in half, lateral regeneration occurs in both the 'halves' with and without the median ganglionic mass (Fig. 3, 5).

5. The difference in locomotion between worms with and without the median ganglion and statocyst, is noticeable within ten days after the cut. The pieces without the sense organ act as 'headless pieces' showing difficulty in righting themselves when overturned, and moving only when mechanically stimulated. As regeneration occurred, these 'headless pieces' gradually approached the normal type in behavior.

6. In another experiment, the anterior end was cut off, and then the rest of the worm cut longitudinally in half. The absence of both median and lateral ganglia does not prevent lateral-anterior regeneration (Fig. 3, 5, 6, 7, 8, 9).

7. If a worm is cut as in Fig. 10 giving two narrow pieces from the sides, regeneration takes place and a small symmetrical worm is formed (Fig. 11). The middle piece forms mostly by reorganization into the normal form (Fig. 12).

8. If the posterior part of an animal is cut in two longitudinally, or cut so that the marginal regions are separated from each other and the wound does not heal, each half or part produces new tissue and forms distinct caudal lobes and filaments. The length of the posterior regenerated region may be controlled by the depth of the cut, or removal of the middle tissue and wound closure. The absence of this middle part does not seem to hinder regeneration (Fig. 14, 17, 18, 24, 26).

9. If the region of the median ganglion is removed, a slight retardation of regeneration occurs, but lateral regeneration is not impaired (Fig. 16).

10. If the cut surfaces do not heal together from a circular cut, or the regenerated tissue does not close the wound within four or five days, then the slit remains open until death (Fig. 28).

11. If the posterior part of a worm is cut as far forward as the

statocyst (Fig. 29, 30), head formation may appear in the notch between the new sides.

12. Reversed grafts between anterior surfaces of *Polychœrus* are readily obtained.

13. If the line of union in reversed grafts is perfect, no regeneration takes place, and locomotion is only possible when one component part of the grafted animal looses its hold on the substrate.

14. When reversed grafts are not perfectly united a head develops from the exposed part. This head may or may not appear to belong to either piece (Fig. 37, 42).

15. If a very small piece is grafted to a larger piece and regeneration occurs in the larger piece but not in the smaller, then absorption of the smaller piece takes place (Fig. 46, 47, 48).

16. When pieces which had grafted together by anterior ends were cut obliquely, the grafted pieces showed morphallaxis and caudal lobes developed from the cut surface (Fig. 51, 52); except in a few cases (Fig. 53). Just which part of the graft regenerated the caudal lobes is not certain, since the detection of the vital dye in the graft was not apparent.

LITERATURE CITED.

Bardeen, C. R.

'01 On the Physiology of the *Planaria maculata*, with Especial Reference to the Phenomena of Regeneration. American Journal of Physiology, 1901, Vol. V., p. 1.

Child, C. M.

'07 The Localization of Different Methods of Form-regulation in *Polychœrus caudatus*. Archiv. für Entwicklungsmechanik der Organismen, 1907, Vol. XXIII., p. 227.

'10 The Central Nervous System as a Factor in the Regeneration of Polyclad *Turbellaria*. Biol. Bull., 1910, Vol. XIX., p. 333.

Délage, Yves.

'86 Études Histologiques sur *Les Planaires Rhabdocœles Acœles* (Convoluta Schultzii-O. Schm.-). Archives de Zoologie Expérimentale et Générale 1886, Vol. XIV., p. 109.

van Duyne, John.

'96 Ueber Heteromorphose bei *Planarien*. Archiv. für die gesammte Physiologie, 1896, Vol. LXIV., p. 569.

Gardiner, E. G.

'95 Early development of *Polychœrus caudatus* Mark. Journal of Morphology, 1895, Vol. XI., p. 155.

'98 The Growth of the Ovum, Formation of the Polar Bodies, and the Fertilization in *Polychœrus caudatus*. Journal of Morphology, 1898, Vol. XV., p. 73.

Graff, L. von

'91 Die Organisation der *Turbellaria acœla*, etc. Leipzig, Engelmann, 1891, 4 to 90, pp., 10 Taf.

Hanström, B.

'28 Vergleichende Anatomie des Nervensystems der Wirsellosen Tiere, 1928.

Joest, Ernst.

'97 Transplantionsversuche an *Lumbriciden*. Archiv. für Entwicklungsmechanik der Organismen, 1897, Vol. V., p. 419.

Lemon, G. C.

'99 Notes on the Physiology of Regeneration of Parts in *Planaria maculata*. Biol. Bull., 1900, Vol. I., p. 193.

Löhner, L.

'10 Untersuchungen über *Polychœrus caudatus* Mark. Zeitschrift für Wissenschaftliche Zoologie, 1910, Vol. XCV., p. 451.

Mark, E. L.

'92 *Polychœrus caudatus:* nov. gen. et. nov. spec. Festschrift zum siebzigsten Geburtstage R. Leuckarts, 1892, p. 298.

Morgan, Lillian V.

'05 Incomplete Anterior Regeneration in the Absence of the Brain in *Leptoplana littoralis*. Biol. Bull., 1905, Vol. IX., p. 187.

'06 Regeneration in Grafted Pieces of *Planarian*. Journal of Experimental Zoölogy, 1906, Vol. III., p. 269.

Morgan, T. H.

'00 Regeneration in *Planarians*. Archiv. für Entwickelungsmechanik der Organismen, 1900, Vol. X., p. 58.

'00 Regeneration in *Bipalium*. Archiv. für Entwickelungsmechanik der Organismen, 1900, Vol. IX., p. 563.

'01 Regeneration, 1901.

Randolph, Harriet.

'97 Observations and Experiments in Regeneration in *Planaria*. Archiv. für Entwicklungsmechanik der Organismen, 1897, Vol. V., p. 365.

Stevens, N. M., and Boring, A. M.

'05 Regeneration in *Polychœrus caudatus*. Journal of Experimental Zoölogy, 1905, Vol. II., p. 335.

A STUDY OF EQUILIBRIUM IN THE SMOOTH DOGFISH (*GALEUS CANIS* MITCHILL) AFTER REMOVAL OF DIFFERENT PARTS OF THE BRAIN.

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In the experiments here reported we have studied equilibrium after removal of

1. End-brain
2. Optic lobes
3. Cerebellum
4. End-brain, optic lobes and cerebellum

In the teleosts, Desmoulius, Vulpian, Flourens and Steiner detected no disturbance of equilibrium after removal of the end-brain. Polimanti noticed that removal of the end-brain caused cessation of motility; even if stimulated with electrical current the animals failed to move. When locomotion did take place it was hesitant and uncertain; equilibrium was disturbed. In the selachians, Steiner found that removal of the end-brain caused immobility for hours and even days. Bethe and Polimanti observed that the animals remained active; equilibrium remained normal.

Baudelot and Vulpian failed to detect disturbances of equilibrium in the teleosts after removal of the roof of the mid-brain. In the selachians, Steiner, Bethe, Loeb obtained similar results; after removal of the optic lobes equilibrium remained normal. Contrary to Baudelot, Vulpian, Steiner, Bethe and Loeb, Polimanti found that removal of the optic lobes in the teleosts and selachians was followed by disturbances of equilibrium.

Renzi and Dickinson observed profound disturbances of equilibrium in the teleosts after removal of the cerebellum (hind-brain). These authors believed the cerebellum to be indispensable for equilibrium. Vulpian and Polimanti noticed that equilibrium remained normal after removal of the cerebellum. In the selachians, Steiner, Bethe, Loeb agreed that removal of the cerebellum produces disturbances of equilibrium. Polimanti's observations concerning the removal of the cerebellum in the selachians did not permit him to deduce conclusions; the results were not decisive. Elsberg and Tilney have found no disturbance of equilibrium after removal of the cerebellum; equilibrium in every respect was comparable to the equilibrium of the normal animal.

Our experiments have been restricted to the selachian (*Galeus Canis*). The size of the animal varied from 50 cm. to 75 cm. Special care was taken to operate while the gills and the anterior portion of the animal's head remained submerged in sea water. The animal was fastened to a cork board inclined at an angle of about 35° in a large dissecting pan. The pan was provided with a continuous flow of sea water.

The different regions of the brain were approached through the roof of the brain case (tegmen cranii). Making first a skin flap, the portion of the cartilaginous encasement above the region to be ablated was removed. After ablation of the particular region, the parts of the brain that remained exposed were covered with cotton. The skin flap was replaced and sutured. All animals died from two to three days after having been operated upon.

The end-brain (cerebral hemispheres and olfactory lobes) was removed by making an incision at the junction (velum transversum) of this part of the brain with the inter-brain (thalamus). Care was taken to injure neither the optic nerves nor the chiasma.

The cerebellum was removed by cutting through its peduncles —the superior and inferior peduncles.

The optic lobes were removed by cutting around the region of each optocœle; to avoid injuring the floor of the mid-brain, precaution was taken not to extend the ablation beyond the optocœles.

Studies of equilibrium were made in an aquarium 2½ meters in length, 1¼ meters in width, 4/5 meter in depth.

DATA.

Removal of the End-Brain.—No disturbance of equilibrium resulted from removal of the end-brain. The animal swam normally in all planes; at the bottom of the aquarium it settled dorsal side up. Placed on its back at the surface of the water, the animal righted itself immediately. Equilibrium was comparable to the equilibrium of the normal animal. Six animals were operated upon.

Removal of the Optic Lobes.—Eight animals were operated upon. In each animal both optic lobes were removed. In three cases the animal swam normally in all planes; in five cases it frequently swam indiscriminately to the right or to the left around the dorso-ventral axis. This disturbance of movement in the horizontal plane cannot be attributed to partial loss of equilibrium because changing the direction of the animal changed the direction of its swimming; turned to the right the animal swam to the right around the dorso-ventral axis; turned to the left, it swam to the left. In these latter cases, movement in the horizontal plane became normal within twenty-four hours after removal of the optic lobes; the tendency to swim around the dorso-ventral axis disappeared. In all eight cases, the animal settled dorsal side up at the bottom of the aquarium. Placed on its back at the surface of the water, the animal righted itself immediately. It failed to avoid the sides of the aquarium.

Removal of the Cerebellum.—The cerebellum was removed in seven animals. The ablation caused no disturbance of equilibrium; the animal swam normally in all planes and settled dorsal side up at the bottom of the aquarium. There was complete absence of rotation around the axes, spirals through the water, nose diving and swimming on its back. Placed ventral side up at the surface of the water the animal righted itself without difficulty; no disturbance of equilibrium was noticed when righting itself.

Removal of the end-brain, optic lobes and cerebellum: Eight animals were operated upon. In each animal the end-brain, the optic lobes and cerebellum were removed. The operative procedure was as follows:

1. The end-brain was removed.
2. The animal was placed in the aquarium for two hours.
3. The optic lobes were removed.
4. Four hours after removal of the optic lobes, the cerebellum was removed in those cases in which movement in the horizontal plane remained normal after removal of the lobes; in the cases in which removal of the optic lobes caused the animal to swim around the dorso-ventral axis, the cerebellum was removed after movement in the horizontal plane had become normal.

No disturbance of equilibrium resulted after completion of all operations. The animal swam normally in all planes but did not avoid the sides of the aquarium. Rotation around the axes, spirals, nose diving and swimming on its back did not occur. The animal was active. Placed on its back at the surface of the water it righted itself immediately and without difficulty. At the bottom of the aquarium it settled dorsal side up.

Conclusion.

In the smooth dogfish, *Galeus Canis*, removal of the end-brain, the optic lobes or the cerebellum does not effect disturbances of equilibrium; equilibrium remains normal even after these three parts of the brain have been removed in the same animal.

BIBLIOGRAPHY.

Bethe, A.

1899 Die locomotion des haifisches (Scyllium) und ihre beziehungen zu den einzelnen gehirntheilen und labyrinth. Pflügers Archiv., LXXVI., 470.

Bottazzi, F.

1895 Il cervello anteriore dei selacei. Labor. Anat. Norm. Roma, IV., 225.

Desmoulins, A.

1822 Sur le rapport le plus probable entre l'organisation du cerveau et ses fonctions. Jour. Compl. du Dict. d. Sc. Med. Paris, XIII., 206.

Desmoulins, A.

1825 Anatomie des systèmes nerveux des animaux a vertèbres applique à la physiologie et à la zoologie. Paris.

Dickinson, W. H.

1865 On the Functions of the Cerebellum. Boston M. and S. Jour., LXXVI. 237.

Dickinson, W. H.

1865 On the Functions of the Cerebellum. Brit. and For. Med. Rev., XXXVI. 455.

Flourens, J. P.

1824 Recherches expérimentales sur les propriétés et les fonctions du système nerveux dans les animaux vertébrés. Paris.

Flourens, J. P.

1825 Experiences sur le système nerveux. Paris.

1844 Mémoire d'anatomie et de physiologie comparées. Paris.

1856. Cours de physiologie comparée. Paris.

Gomez Ocaña, J.

1908 Contribución al estudio de las funciónes de las lóbulos ópticos de los peces. Bol. Soc. Española. Hist. Nat., VIII., 247.

Polimanti, O.

1913 Contributions à la physiologie du système nerveux central et du mouvements des poissons. Archiv. Ital. Biol., LIX., 383.

Renzi, P.

1857 Riflessioni e spermenti per servire di materiale alla fisiologia del cervelletto. Gazz. Med. Ital. Lomb., Milan, 4 s., II, pp. 266, 342, 425.

Renzi, P.

1858 Riflessioni e spermenti per servire di materiale alla fisiologia del cervelletto Gazz. Med. Ital. Lomb., Milan, 4 s., III, pp. 35, 112, 196, 265, 343, 416.

Renzi, P., and Morganti, G.

1859 Riflessioni e spermenti per servire di materiale alla fisiologia del cervelletto. Gazz. Med. Ital. Lomb., Milan, 4 s., IV., pp. 44, 113.

Steiner, J.

1886 Ueber das centralnervensystem des haifisches und des amphioxus und über die halbzirkelförmigen canäle des haifischen. Sitzb. d. König. Preuss. Akad. d. Wiss. Berlin, I., 495.

Steiner, J.

1888 Die functionen des centralnervensystem und ihre phylogenese: die fishe. Braunschweig, 112.

Tilney, F.

1923 The Genesis of Cerebellar Functions. Archiv. of Neur. and Psych., IX., 137.

Vulpian, A.

1866 Physiologie du système nerveux. Paris.

HETEROAGGLUTINATION OF DISSOCIATED SPONGE CELLS.

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It has been shown in my previous papers (Galtsoff, 1923, 1925) that in a suspension of artificially separated tissue cells of *Microciona prolifera* the amœboid movement of the archæocytes is instrumental in the formation of cell aggregates and that the movement of the archæocytes is inhibited by the presence of the cells of another species. The inhibitive effect of the foreign cells is apparently due to the substances excreted by them or, more probably, liberated from those foreign cells which were injured in the preparation of the suspension.

Further observations disclosed the fact that when in a compound suspension of *Microciona* and *Cliona* the archæocytes of two sponges come together, the outer hyaline layers of their protoplasms fail to coalesce, the cells of each species remaining separated and forming aggregates of its own kind. There is no doubt that such a segregation is due to the difference in the physical properties of the outer protoplasmic layers of the cells of two species. It was the author's intention to continue a study of this phenomenon on a greater variety of sponges. Thanks to the generous grant of the American Association for the Advancement of Science, the author was able to carry out the experiments at the Bermuda Biological Station in June–July 1925. The publication of the results has been delayed on account of the difficulty of identifying some of the Bermuda sponges and because of other investigations in which the author was engaged. It is the privilege of the author to express his thanks to the A. A. A. S. for a grant which made this work possible; to Dr. E. L. Mark, Director of the Bermuda Biological Station, for the permission to occupy a laboratory space; and to Dr. H. V. Brφndsted, Birkerod, Denmark, for the identification of six species of sponges.

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MATERIAL AND METHOD.

All the sponges used in the experiments were collected in the shallow water around Agar's Island. Previous experiments with *Microciona* and *Cliona* had shown that sponges do not keep well in laboratory aquaria and that after a few days of confinement their regenerative ability is greatly reduced. Because of the necessity of having an abundant supply of fresh material always at hand the selection of species has been governed by their abundance and accessibility. The following siliceous and horny sponges were used:

Reniera cinerea Grant; *Pachychalina* sp.; *Spinosella sororia* Dendy; *Tedania ignis* D. and M.; *Donatia* sp.; *Suberites carnosus* Johnston; *Aplysina crassa* Hyatt; *Aplysina hirsuta* Hyatt; *Euspongia irregularis* L. J. Lendenfeld.

Reniera, *Pachychalina*, *Spinosella* and *Tedania* belong to the family of Haplosceridæ; *Suberites* and *Donatia* are members of the families Suberitidæ and Donatidæ respectively; *Euspongia* and *Aplysina* are horny sponges belonging to the family Spongiidæ. The species of *Pachychalina* used in the experiments is characterized by its bright scarlet color. According to Brφndsted it does not belong to any of the previously described species.

The method employed in the present investigation was essentially the same as was used in previous work (Galtsoff, 1925). After rinsing the sponges in filtered sea water and removing the leaves of algæ and other foreign material, small pieces weighing 10 grams, were squeezed through bolting silk No. 20 into 100 ccm. of filtered sea water poured into a finger bowl or a crystallizing dish. Perfectly clean slides were placed on the bottom of the dish and the cells were allowed to settle on them. The slides were examined at regular intervals. For a study of agglutination, undiluted cell suspension of a given species was poured on a slide and mixed with a known amount of suspension of another sponge. The temperature of the water varied from 26° to 28° C.; the salinity of the water computed from hydrometer readings fluctuated from 29.5 to 31.5 parts per thousand.

Behavior of the Dissociated Cells.

Similar to the behavior of *Microciona* cells described by Wilson (1911) and Galtsoff (1925) the separated cells of each of the species of Bermuda sponges tested during the present investigation displayed amœboid movement and upon coalescence with each other formed aggregates. There was, however, a considerable difference in the velocity and duration of the amœboid movement of the cells of various species and, consequently, in the size and number of their aggregates. The most active amœboid movement resulting in the formation of a few large globular aggregates was displayed by the cells of *Reniera cinerea* and *Pachychalina*, whereas the cells of *Donatia* and *Spinosella* remained almost inactive and even after several hours following dissociation formed but very small and loose aggregates comprising a few cells. According to the ability of the separated cells to reunite, the sponges used in these experiments can be arranged in the following inequality series, beginning with the species which displayed the most active amœboid movement: *Pachychalina* sp. > *Reniera cinerea* > *Tedania ignis* > *Aplysina crassa* > *Aplysina hirsuta* > *Euspongia irregularis* > *Suberites carnosus* > *Donatia* sp. > *Spinosella*. The initial steps in aggregate formation by the dissociated cells of *Pachychalina*, *Reniera* and *Tedania* are similar to those observed in *Microciona*. The cells settle on the bottom forming a network of coalesced material, which gradually contracts and transforms into a number of round aggregates. During this period the archæocytes display an active amœboid movement forming large hyaline pseudopods and coalescing with one another and with other tissue cells, the amœboid movement of which is much slower. Unlike *Microciona* the cell aggregates of Bermuda sponges fail to form a firm attachment to glass or other solid surfaces (shells, rocks, celluloid, leaves of algæ). After a period varying from 12 to 24 hours they begin to curl up and detach themselves from the substratum. No further development has been noticed in the detached sphæroidal masses, which were kept for several days in the sea water; they all died within a few days. Similar difficulties were encountered by Wilson (1925) in his experiments with several species of *Pachychalina* at Tortugas. De Laubenfels (1926, 1928) working on the same material, succeeded, however, in overcoming this obstacle, which he thinks was due to bacterial growth; he was able to keep the conglomerates of sponge cells at least two weeks, during which time they developed into functional sponges. Numerous attempts made in the course of the present investigation to prevent the detachment of aggregates by changing the pH of the water and by increasing the calcium content of it were unsuccessful. In spite of all efforts the separation of aggregates from the substratum occurred within 24 hours.

As has been stated above, the most active amœboid movement and aggregate formation occurs in the suspension of cells of *Reniera cinerea* and *Pachychalina*. *Reniera* suspension comprises a large number of archæocytes, 12–15 micra in diameter, loaded with large greenish granules. Less abundant are small round cells, 5–6 micra in diameter, containing few granules, and other cells of approximately the same size but entirely devoid of any protoplasmic inclusions. *Choanocytes* are very abundant and very active; by the beating of their cilia they agitate the suspended material and push around the cells which, coming in contact with the archæocytes, stick to their protoplasm and coalesce. In this manner the choanocytes play an important rôle in the reunion of separated cells. The aggregates of reunited cells continue to move over the substratum and coalesce with one another and with single cells that happen to lie on their route. The single archæocytes and the aggregates move in typical spiral paths (Galtsoff, 1923, 1925). In a few hours the amœboid movement ceases completely. By this time the coalesced cells have formed rounded aggregates, consisting of tightly packed cells, and have attached themselves to the substratum. The size and the number of aggregates found over a given area and formed under similar conditions can be used as a relative index of the duration and velocity of their movement. The slower the latter, and the shorter its duration, the greater is the number of aggregates and the smaller their diameter. In all the sponges tested, the process of the reunion of the separated cells is essentially similar to that just described, the only difference being in the velocity and duration of the amœboid movement. It is therefore unnecessary to give a separate description for each species of sponge.

HETEROAGGLUTINATION OF SEPARATED CELLS.

Similar to the conditions found in a compound suspension of *Microciona* and *Cliona* cells the presence of foreign cells inhibits the amœboid movement of *Reniera* archæocytes and prevents their aggregation. This can be seen in the suspensions of *Reniera* and *Pachychalina* cells. The suspensions were prepared by squeezing 10 grams of sponge into 100 ccm. of sea water; 2 ccm. of suspension were added to 8 ccm. of sea water poured into Syracuse glasses. Various amounts of *Pachychalina* suspensions were added to *Reniera* suspension (Table 1). After three hours all the dishes were examined and the average number of aggregates for a given area (1.16 sq. mm.) was computed.

TABLE 1.

THE FORMATION OF AGGREGATES IN A COMPOUND SUSPENSION OF *Reniera* AND *Pachychalina* CELLS. (R—*Reniera*, P—*Pachychalina*, SW—sea water.)

Suspension.	Average Number of Aggregates.	Limits.	Number of Squares Examined.
2.0 R + 8.0 SW	7.9	5–12	20
2.0 R + 8.0 SW	9.6	4–16	20
2.0 P + 8.0 SW	5.0	3–7	20
2.0 P + 8.0 SW	5.9	3–9	20
2.0 R + 2.0 P + 6.0 SW	0		50
2.0 R + 1.0 P + 7.0 SW	0		50
2.0 R + 0.5 P + 7.5 SW	0		50

Microscopic examination of a compound suspension of *Reniera* and *Pachychalina* cells reveals that immediately upon the addition of foreign cells the amœboid movement of Reniera archæocytes is completely inhibited and that the beating of the flagella of *Reniera* choanocytes stops. The effect of *Reniera* cells on *Pachychalina* is even more pronounced; in a few seconds after Reniera cells are added to *Pachychalina* suspension the cells of the latter undergo rapid irreversible changes. The outer layer of the archæocytes bursts, the cells lose their regular shape;

the granuloplasm flows out through the injured part of the cell body and the cells undergo complete or partial cytolysis. This phenomenon is accompanied by an immediate agglutination of the suspended material, which forms large, loose flocculi settling on the bottom but failing to adhere to it. In less than half an hour the cells of *Pachychalina* are completely cytolized, while most of the *Reniera* cells remain alive but motionless. The agglutinated material perishes in a few hours.

The phenomenon of heteroagglutination occurs in several sponges and is essentially similar to that just described. It is most pronounced in *Reniera cinerea*, which agglutinates the cells of all other sponges tried in the present investigation. The results of all the experiments are summarized in table 2 in which the positive and negative agglutination reactions are denoted by + and − respectively. Several conclusions can be drawn from the examination of this table. It is shown that the heteroagglutination of separated cells is not always reciprocal. For instance, *Reniera cinerea*, which agglutinates the cells of all other sponges, is in turn agglutinated by *Tedania ignis* and *Aplysina crassa*, but is not agglutinated by *Pachychalina*. *Tedania* is agglutinated by *Aplysina hirsuta*, but the latter is not agglutinated by *Tedania*. *Pachychalina* agglutinates the cells of *Aplysina hirsuta* and is agglutinated by the latter; *Aplysina hirsuta* agglutinates *Pachychalina*, *Tedania* and *Spinosella; Euspongia irregularis* agglutinates *Spinosella* and is agglutinated by the latter; *Spinosella* agglutinates *Aplysina hirsuta* and *Euspongia irregularis* and is in turn agglutinated by *Euspongia* and *Aplysina hirsuta*. The determination as to which cells are agglutinated and which remain free is possible when the cells of two sponges can be recognized by their respective colors, or when the reaction is induced by the addition of a very small amount of agglutinating suspension. The formation of large flocculi and clarification of the turbid fluid can be observed with the naked eye.

In the majority of cases the reaction takes place immediately upon the addition of agglutinating suspension, but in a few instances from two to five minutes elapse before the formation of flocculi becomes noticeable.

The efficacy of the agglutinating suspension can be estimated by determining the minimum dose which causes the reaction. The differences in the agglutinability and agglutinating power of various sponges can be seen in table 3, showing the ratios between the minimum volumes of agglutinating and agglutinated suspensions involved in the reaction.

TABLE 2.

HETEROAGGLUTINATION OF DISSOCIATED SPONGE CELLS.

Sponge Added.	Sponge Agglutinated.								
	Reniera cinerea.	*Pachychalina* sp.	*Spinosella sororia.*	*Tedania ignis.*	*Donatia* sp.	*Suberites carnosus.*	*Aplysina crassa.*	*Aplysina hirsuta.*	*Euspongia irregularis.*
Reniera cinerea	−	+	+	+	+	+	+	+	+
Pachychalina sp.	−	−	−	−			−	+	−
Spinosella sororia		−	−	−			−	+	+
Tedania ignis	+	−	−	−		−		−	−
Donatia sp.	−				−				
Suberites carnosus						−			−
Aplysina crassa	+	−	−	+			−	−	−
Aplysina hirsuta		+	+	+			−	−	
Euspongia irregularis		−	+	−			−	−	−

TABLE 3.

THE AGGLUTINABILITY OF VARIOUS SUSPENSIONS OF SPONGE CELLS.

Sponge Added.	Sponge Agglutinated.								
	Reniera cinerea.	*Pachychalina* sp.	*Spinosella sororia.*	*Tedania ignis.*	*Donatia* sp.	*Suberites carnosus.*	*Aplysina crassa.*	*Aplysina hirsuta.*	*Euspongia irregularis.*
Reniera cinerea		1 : 25	1 : 10	1 : 50	1 : 25	1 : 25	1 : 25	1 : 25	1 : 20
Pachychalina sp.								1 : 5	
Spinosella sororia								1 : 10	1 : 10
Tedania ignis	1 : 5								
Aplysina crassa	1 : 5			1 : 5					
Aplysina hirsuta		1 : 5	1 : 1	1 : 1					
Euspongia irregularis			1 : 1						

As can be noticed from the examination of this table, the agglutination occurs in certain instances (*Reniera* vs. *Tedania*) when one drop of agglutinating suspension is added to fifty drops of agglutinable material, while in other cases (*Euspongia* vs. *Spinosella; Aplysina hirsuta* vs. *Spinosella*) equal volumes of two suspensions should be mixed to cause a positive reaction. It is interesting to note that *Reniera* agglutinates with almost equal readiness the cells of *Pachychalina*, which belongs to the same family of Haplosclerid*æ*, and the cells of *Aplysinæ* and *Euspongia* belonging to a different order (Keratosa). On the other hand *Tedania ignis* is agglutinated more readily by *Reniera* than are the cells of the other genera of the family Haploscleridæ. Apparently the ability of sponges to agglutinate the cells of another genus cannot be correlated with their taxonomic position. Attention should be directed, however, to the fact that agglutination reaction fails to take place when the cells of two species of genus *Aplysina* are mixed together.

It has been found in the experiments with *Reniera* that the substance responsible for the agglutination of cells is soluble in sea water. The suspension of *Reniera* cells was filtered and centrifuged for 15 minutes at the rate of 1,200 revolutions per minute. The efficacy of clear supernatant fluid in inducing agglutination was equal to that of the unfiltered suspension.

It is known that, similar to the behavior of suspensions of inert particles, the suspensions of certain live cells (bacteria, red blood corpuscles) become unstable and agglutinate at the isoelectric point of the solution in which they are kept (Fauré-Fremiet et Nichita, 1927; Girard, 1912). On the other hand Coulter (1921), Eggerth (1924) and Winslow, Falk and Caulfield (1923) have shown that by acidifying the medium in which the cells are kept in suspension it is possible to neutralize the negative charges of the suspended particles. It is therefore possible to suppose that the agglutination in sponge cells may be due to the changes in the electric charges of the cells brought about by the changes in the hydrogen ion concentration of the surrounding medium. Crozier (1918), working on one of the Bermuda *Aplysinæ*, which he called Sponge A and which answers the description of *Aplysina crassa*, has demonstrated that the intracellular acidity of this sponge is about pH 6.0. It has been noticed during the present investigation that the bright yellow color of the interior portion of the *Aplysina* tissues turns purple upon exposing it to sea water of the pH 8.2; the cells of the interior portion of the colony kept in acid sea water (pH 4.4–5.0) remain yellow. The conclusion seems inevitable that the tissues of *Aplysina crassa*, except those located on the surface of the sponge, are more acid than the surrounding sea water. Unfortunately other sponges used in the experiments do not possess pigments which can be used as indicators. In the light of work by Galtsoff and Pertzoff (1926), which has demonstrated that the suspensions of *Microciona* and *Cliona* cells behave either as weak bases or weak acids depending on the hydrogen ion activity of the suspension, it is reasonable to suppose that similar conditions exist in all siliceous sponges. One should expect therefore that the suspensions of sponge cells in sea water will decrease the pH value of the latter. As previous experiments have shown (Galtsoff and Pertzoff, 1926), the establishment of the new equilibrium between the cells and the surrounding medium takes a certain length of time and is dependent upon the number of cells in suspension. Several determinations of the pH values of the suspensions of Bermuda sponges, prepared under standard conditions (approximately equal number of cells in suspension; pH of sea water 8.2; temperature 26–28°), show that in ten minutes after the preparation of the suspension the reaction of the latter was acid (table 4).

TABLE 4.

pH VALUES OF THE SUSPENSIONS OF SPONGE CELLS.

	pH
Sea water	8.2
Reniera cinerea	6.4
Pachychalina sp.	6.0
Aplysina hirsuta	6.0–6.3
Aplysina crassa	6.6

Table 4 shows that there are no significant differences in the pH values of the suspensions which can be correlated with their agglutinating power and that apparently the agglutination of dissociated cells is not influenced by the increase in the hydrogen ion concentration. Further support of this conclusion is found in the fact that the addition of 1/100 N HCl does not cause the agglutination of the suspended material. In this respect the dissociated sponge cells are similar to the amœbocytes of *Arenicola* and *Asterias*, the agglutination of which, as has been shown by Fauré-Fremiet (1927) is independent of the isoelectric point and above a certain lowest value occurs at any given pH. The conclusion can be reached that the agglutination of dissociated sponge cells is brought about by some substance or substances present in a suspension of cells, which cause the irreversible changes in the physical properties of the cell membrane resulting in breaking up of the cell body, efflux of the protoplasm, and aggregation of the suspended material. The nature of this substance is unknown.

BIBLIOGRAPHY.

Coulter, C. B.

'21 The Isoelectric Point of Red Blood Cells and Its Relation to Agglutination. The Journal of General Physiology, Vol. 3, p. 309–323.

Crozier, W. J.

'18 On Indicators in Animal Tissues. The Journal of Biological Chemistry, Vol. XXXV., No. 3, p. 455–460.

de Laubenfels, M. W.

'26 Bi-specific Conglomerations of Sponges. Carnegie Institution of Washington, Year Book No. 26, pp. 219–222.

de Laubenfels, M. W.

'28 Interspecific Grafting, Using Sponge Cells. Journal of the Elisha Mitchell Scientific Society, Vol. 44, pp. 82–86.

Fauré-Fremiet, E., et Nichita, G.

'27 Charge électrique et agglutination chez les amibocytes d'Invertébres marins. Annales de Physiologie et de Physico-chimie Biologique, 1927, No. 2, p. 247.

Galtsoff, P. S.

'23 Amœboid Movement of Dissociated Sponge Cells. BIOL. BULL., Vol. 45, pp. 153–161.

Galtsoff, P. S.

'25 Regeneration after Dissociation (an Experimental Study on Sponges). I. Behavior of Dissociated Cells of *Microciona prolifera* under Normal and Altered Conditions. Journal Experimental Zoölogy, Vol. 42, pp. 183–221.

Galtsoff, P. S., and Pertzoff, V.

'26 Some Physico-chemical Properties of Dissociated Sponge Cells. The Journal of General Physiology, Vol. X., No. 2, pp. 239–255.

Girard, P.

'12 Sur la charge électrique des globules rouges du sang. C. R. Acad. Sc. CLV., pp. 308–310.

Wilson, H. V.

'07 On Some Phenomena of Coalescence and Regeneration in Sponges. The Journal of Experimental Zoölogy, Vol. V., No. 2, pp. 245–258.

Wilson, H. V.

'25 Studies on Dissociated Sponge Cells. Carnegie Institution of Washington, Year Book No. 24, pp. 242–246.

Winslow, C. E., Falk, I. S., and Caulfield, M. F.

'23 Electrophoresis of Bacteria as Influenced by Hydrogen-ion Concentration and the Presence of Sodium and Calcium Salts. The Journal of General Physiology, Vol. VI., pp. 177–200.

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BIOLOGICAL BULLETIN

EGG LAYING HABITS OF *GONIONEMUS MURBACHII* IN RELATION TO LIGHT.[1]

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According to H. F. Perkins (*Proc. Philadelphia Acad. Sc.*, 1902) *Gonionemus murbachii* lays eggs only at night or in artificial darkness during the day and egg-laying can be induced more easily in the afternoon than earlier in the day. The tissues "get ready" for dehiscence of eggs within one minute after the animal is placed in the dark and the constancy of egg-laying is not affected by temperature.

It is the purpose of this paper to discuss some additional quantitative data which has been secured in regard to the egg-laying habits of *Gonionemus murbachii* and the effect of vital stains on these habits. The following data are to be used as control observations in an attempt to determine which part (or parts) of the visible spectrum is responsible for this spawning reaction. Report of these further experiments will follow.

Method

Gonionemus murbachii lays eggs which, very shortly after being shed, will sink to the bottom and by means of a sticky surface will adhere to any objects with which they come into contact. The eggs are shed by vigorous contractions of the bell (Perkins) and since the animal, by these contractions, stirs up the water in which the eggs are laid, even distribution of the eggs is insured.

Crystallizing dishes measuring 7½ cm. deep and 13.5 cm. in diameter and having a capacity of one liter were used. The bottom surface of these dishes was calculated to be 143 square centimeters. In each dish were placed three glass slides which had previously been marked off with a glass cutter into twelve square centimeters each. This gave a total of thirty-six square centimeters of bottom surface that were clearly indicated. The slides were so placed in the dishes as to be at right angles to each other and to form a wide figure "H." Careful examination of a number of dishes containing eggs was made and it was concluded that the average of the thirty-six square centimeters of bottom surface that was clearly marked off would give an accurate basis for estimaion of the total number of eggs on the 143 square centimeters of bottom surface.

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[1] To Dr. C. G. Rogers of Oberlin I am indebted for the original introduction to the problems herein involved.

The animals were collected between 8:30 and 11:30 A.M. and were used the same day. Only the largest individuals were chosen, the females being identified under the low power of the microscope. A female would be placed in a crystallizing dish to which had been added 850 cc. of fresh sea water, sea lettuce and scrapings of diatoms from eel grass (food). Water, slides, crystallizing dish, and food were changed for each female after each reading and a large photographic dark room was used for confinement. Each dish was covered by a glass plate to prevent evaporation and temperature readings were taken at the beginning and end of each designated period.

Data and Discussion.

The control individual (Female 7-10-*A*) was kept under observation for a period of eleven days during which time it laid 75,579 eggs, or an average of 6872 eggs per night. The extreme variations in number were 4,380 and 9,387, the higher number being laid toward the end of the observation period. This individual was exposed to natural light from 9:30 A.M. until darkness when it was placed in artificial light until 9:30 P.M. every night. From 9:30P.M. until 9:30 A.M. it was confined in the dark room. During the day it was placed for a very short period in direct sunlight. Fresh sea lettuce, sea water and food were placed in the dish daily, as was also the case with all of the experimental animals. This individual laid no eggs during exposure to light, whether daylight or artificial light, but laid eggs very consistently during the dark confinement at night

The case history of Female 7-10-*G* indicates several unique situations and warrants a record of its history here. This female was watched for a period of fifteen days during which it laid 134,280 eggs or an average of 8,952 per day. After several days of observation, the individual was confined to the dark for a period of 44½ hours following which it was exposed to light for 13 hours and again confined to the dark. The result was that this female laid the greatest number of eggs in a single night recorded in the whole series of observations, namely 22,165 eggs. This may represent a "piling up" situation, due in part to long confinement in the dark followed by a normal period in light. The history reads as follows:

9:30 P.M. (D.)— 9:30 A.M.—10,560
9:30 A.M. (L.)— 9:30 P.M.—none
9:30 P.M. (D.)— 9:30 A.M.—11,466
9:30 A.M. (L.)—11:30 A.M.—none
11:30 A.M. (D.)— 7:00 P.M.—11,726
7:00 P.M. (L.)— 7:30 P.M.—none
7:30 P.M. (D.)— 8:30 A.M.—4719
8:30 A.M. (L.)— 9:30 A.M.—none
9:30 A.M. (D.)—11:30 A.M.—14,157
11:30 A.M. (D.)— 2:00 P.M.—none
2:00 P.M. (D.)— 5:00 P.M.—none
5:00 P.M. (D.)— 9:30 P.M.—none
9:30 P.M. (D.)— 8:30 A.M.—none
8:30 A.M. (L.)— 9:30 P.M.—none
9:30 P.M. (D.)— 9:30 A.M.—*22,165*
9:30 A.M. (L.)— 9:30 P.M.—none
9:30 P.M. (D.)— 9:30 A.M.—9004
9:30 A.M. (L.)— 9:30 P.M.—none
9:30 P.M. (D.)— 9:30 A.M.—5720, etc.

One further case may be reported here as being an exception to the general summary of the paper, though the "exception" can be adequately explained. This is the only case among the thirty-nine histories recorded in which any eggs were laid in the light. It seems probable that this was due to a sudden interruption of the normal egg laying period by bringing the individual into the light half an hour after having been placed in the dark. The eggs that were laid probably represented those which would have been shed with the next several contractions of the bell. This contention is somewhat supported by the fact that the number of eggs shed is relatively very small. In any case, this seems to indicate that all of the eggs are not laid immediately upon confinement in the dark, as was suggested by Dr. Perkins.

Female 7-17-*O*

9:30 P.M. (D.)— 9:30 A.M.—5722
9:30 A.M. (L.)— 9:30 P.M.—none
9:30 P.M. (D.)—10:00 P.M.—4576
10:00 P.M. (L.)— 9:30 A.M.—1431
9:30 A.M. (L.)— 9:30 P.M.—none
9:30 P.M. (D.)— 9:30 A.M.—8500, etc.

Summary and Conclusions.

1. *Gonionemus murbachii* never lays eggs in the light under normal environmental conditions.

2. The only occasion upon which eggs can be induced in the light is when the active egg-laying period (during the hour after confinement in the dark) is suddenly interrupted. Apparently the animal cannot interrupt the shedding of eggs that have been partly extruded, and the "irritation" of exposure to light probably causes the contractions of the bell to be somewhat more vigorous.

3. Eggs are never laid in the light even after prolonged exposure to light during the normal egg-laying season.

4. It is quite possible that *G. murbachii* in its normal habitat lays eggs during dusk rather than in total darkness and not in merely a few contractions of the bell as suggested by Perkins Natural darkness is, of course, gradual, while the experimental confinement in darkness is sudden.

5. Light is probably necessary for the maturation of the egg cells of *G. murbachii.*

6. Eggs will always be laid at night except under the following conditions:

(*a*) When exposed at night to artificial light.
(*b*) When kept in darkness since the preceding egg laying period.
(*c*) When placed in darkness for one hour or more during the

preceding afternoon. In this case eggs may be laid during the night but will represent the balance between the number laid in the afternoon and the number that would normally have been laid during the night.

7. At the height of the egg laying period there is a definite number of eggs that, under normal conditions, are ready for dehiscence every night. Some histories showed a variation upward in number of eggs laid during consecutive nights. These eggs may be laid:

(*a*) During the first hour of darkness following exposure to light (daylight or artificial light).

(*b*) During the afternoon in artificial darkness providing the eggs have not been shed too recently before confinement *or* the animal has been exposed to at least an hour of light preceding the confinement.

(*c*) During the morning in artificial darkness if the previous night's supply has been held back, entirely or in part, by exposure of the animals to light during the night.

8. There is a physiological limit to the number of mature eggs which can be accumulated in light for a particular animal. There is, therefore, no correlation between the length of light exposure and the number of eggs matured.

9. The animals do not measure over 2½ centimeters in diameter, hence the maximum of 22,165 is an almost incredible number of eggs for an individual to shed during one hour of confinement in the dark. An individual with six rays (gonads) laid fewer eggs than most of the four-rayed individuals, but there may have been undetectable maturity differences.

10. Thirty-nine *Gonionemi* representing 127 nights laid a total of 843,510 eggs or an average of 6,642 eggs per night per single jellyfish. These thirty-nine individuals had been selected as being relatively mature females.

11. Slight variations in temperature and pH which occur under normal experimental conditions have no correlation whatever with the numbers of eggs layed.

12. The commonly used vital stains, methylene blue, and neutral red, have, at the most, only a transient effect upon the number of eggs laid. If the number is reduced immediately after

staining it is made up during the subsequent egg laying period. There is the possibility that these vital stains may have some stimulating effect on the egg laying process. (A report is elsewhere to appear giving data to indicate that these vital stains increase the vitality and prolong the life of specimens of *G. murbachii.*) The stain may in some way cut out that part of the visible spectrum which inhibits normal egg laying.

The above observations indicate that there is a definite correlation between the egg laying habits of *Gonionemus murbachii* and light, whether the light is natural or artificial. Vital stains have at the most only a transitory effect upon these regular egg laying habits and these effects may be due in part to the light transmitting qualities of these stains. This leads us to the proposition that further experiments are in order which will determine the effects of various parts of the visible spectrum on these egg laying habits as well as the effects of other factors such as food variations and presence of the male. A few casual observations indicated that presence of the male was not sufficient to induce egg laying in light, or even to increase markedly the number of eggs laid. It is hoped that these further experiments will soon follow.

SOME OBSERVATIONS ON SPERM DIMORPHISM.

GERARD L. MOENCH, M.D., F.A.C.S., AND HELEN HOLT, B.S.

In recent investigations[1] of the fertility of about 150 men we studied first the morphology of the semen, and then calibrated accurately in 0.50 mm. the head lengths of the spermatozoa. The technique was the usual one for such studies; projection onto a screen of the image of the cell at a known definite magnification which was 3,000 diameters in our work. Such studies have been made before, but previous to the work of Williams and Savage (1–5 inclusive), measurements of the head lengths of mammalian spermatozoa were mostly only concerned with the question of dimorphism, and a certain large number of head lengths accurately calibrated at a sufficient magnification when arranged in a frequency distribution polygon were supposed to result in a dimodal graph. Thus Wodsedalek (6, 7) claimed such dimodalism resulted from measuring the sperm head length of the pig, horse, and bull. Zeleny and Faust (8) obtained similar results in the case of the ram, dog, and bull, and Parkes (9) in man, the rat and mouse, and perhaps the cat. It was therefore thought that two types of spermatozoa existed, one producing female and the other male offspring, as the difference in size of the two groups was explained by the presence or absence of the odd or sex-determining chromosome. However, Hance (10), working with the pig, has shown that the chromosomes, forty in number, are constant, but may vary as much as 12 per cent. in the spermatogonal pairs. Any definite division into group sizes on the basis of this finding must, however, be purely accidental. Williams and Savage (1, 2, 3), in their work with bull sperms, never found any dimodalism in normal animals. On the contrary, the closer the frequency polygon approached a normal frequency distribution, the better in most cases the reproductive fitness of the animal turned out to be—the cœfficient of variability especially being that function of the frequency distribution of the population

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[1] With financial assistance from the New York Committee of Maternal Health.

which formed an indicator as to the animal's fertility. Thus, the fertility decreased as the cœfficient of variability increased. No semen specimen, of course, shows all the sperms to be of the same size, any more than all the cells are ever morphologically perfect. At the same time, Williams and Savage found that the sperm heads from one ejaculate did not vary beyond reasonable limits in normal cases.

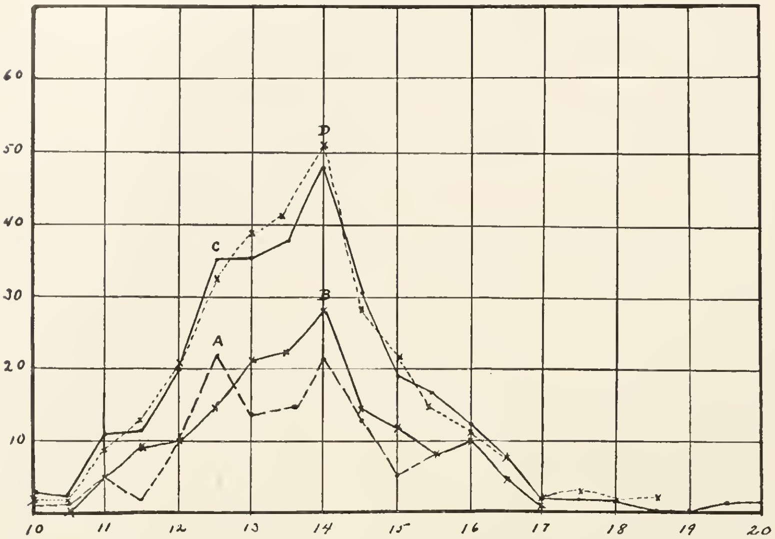

Fig. 1. Case No. 13, *B*. This case represents two graphs *C* and *D* from calibrations of 300 cells each on the same specimen, and, except for some minor changes, the two frequency polyhedrons have approximately the same shape and mathematically it can be shown that the differences between the two curves as compared to the probability of error is not significant and not greater than may be expected from random sampling. Repeated graphs of this kind were frequently made to determine the accuracy of our methods and to compare the accuracy of various methods of calibration. *A* represents first 131 cells of graph *C*; *B* second 169 cells of graph *C*; *C* equals combination of *A* and *B*. See also text.

In this graph, as in the other one here reproduced, the figures on the ordinate or vertical line give the number of heads of any particular length observed, whereas the figures on the abscissa, or horizontal line, give the size of the heads in millimeters (and half millimeters) at a magnification of 3,000 diameters.

In our work with human spermatozoa we can to date but corroborate the conclusions of Williams and Savage. Never, even in our abnormal cases, did we see any evidence of dimorphism of the sperm head, although we were constantly on the lookout for it. The nearest approach to a dimodal graph obtained in our series of cases in shown in Fig. 1. Here the first 131 cell heads measured gave a distinctly dimodal graph (Curve *A*), yet the curve straightened out in the next 169 cells, or for the total of 300 cells usually measured on each specimen.

The failure of Williams and Savage and ourselves to find sperm dimorphism in the bull and man, where it had been described before by others, must have some explanation. Perhaps some of the discrepancy present may be explained by the unit of measurement employed. Some observers used 0.25 mm. as a class unit, whereas both Williams and Savage and we used 0.50 mm. Since bull sperms are about twice as large as human sperms, 0.50 in our work really represents twice as coarse a scale, so that at first when trying out various methods to determine the best way to calibrate human sperm heads we also used a 0.25 mm. scale on a number of cases. We soon found out, however, that this class unit was so small, and the resulting minute differences of measurement so hard to judge that the results, instead of being more accurate, became less so. As a result, some of the cases calibrated with this unit of measurement showed an apparent sperm dimorphism-which, however, was not really present, since it disappeared when we re-measured these same specimens with a 0.5 mm. scale, It might seem to some, therefore, that our scale of 0.5 mm. was too coarse to show up dimorphism. This, however, must be denied, since the claimed differences in size of the two groups of sperm heads was always a number of times larger than our class unit. Hence it would seem to us, though it may sound temerarious to say so, that some of the reported instances of sperm head dimorphism were due to errors in the methods employed. This opinion would seem to receive corroboration from the work of Lush (11), who found much less dimorphism of the boar sperms than was claimed by Wodsedalek (6, 7). We have also had occasion to measure the sperm head lengths of the boar, and Fig. 2. shows the graph obtained by measuring 300 heads of a normal fertile boar. The beautiful symmetrical and normal distribution is at once apparent in this figure. Of course one positive observation is not contraindicated by any number of negative findings, still the hundreds of cases examined by Williams and Savage and ourselves presenting no sperm dimorphism offer strong evidence that such dimorphism must at least be the exception rather than the rule. It must also be remembered that the material used by the various workers in this field was not always the same. While Williams and we employed ejaculated sperm cells, Wodsedalek made sections of the testicle and measured those spermatozoa which were free in the lumina of the tubules, since he considered these cells mature. It has been pointed out before that a number of observers believe that the sperms first mature in the epididymis. Furthermore, histological sections have gone through so many processes that one cannot tell how the spermatozoa may have

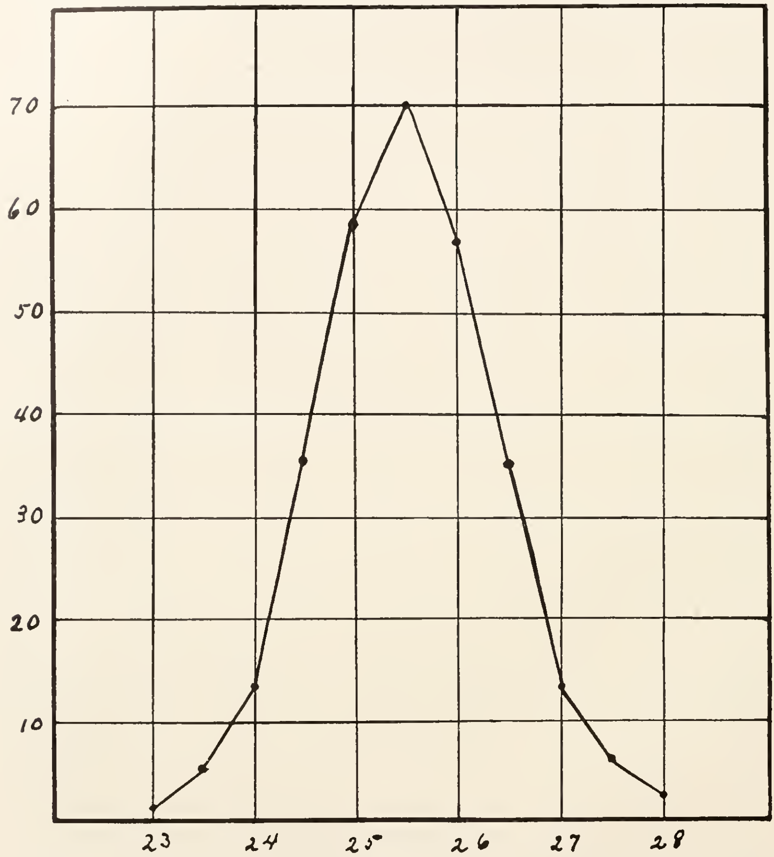

Fig. 2. This graph is the result of measuring 300 sperm head lengths of a known normally fertile boar.

been affected. For this reason the claims set up for sperm dimorphism by Wodsedalek are at least subject to grave doubts, and any size class differences found may have been produced by pure accident.

The results obtained by Zeleny and Faust also seem to us fortuitous, and are not comparable to our investigations, since these observers measured only the basal, usually darker stained portion of the cell, as this only was supposed to represent the nuclear material. With the ever-increasing proof that the whole sperm head consists of nuclear material, Zeleny and Faust's work loses in importance, since no reason seems assignable why the length of the anterior, lighter portion of the sperm head probably representing the head cap should influence in any way the fertilizing power of the cell.

REFERENCES.

1. **Williams and Savage.** Cornell Vet., Oct., 1925.
2. **Williams and Savage.** Cornell Vet., p. 162, 1926.
3. **Williams and Savage.** Trans. Roy. Soc., Canada, Third Series, Vol. 21, Section V, 1927.
4. **Williams and Savage.** J. A. V. M. A., Vol. 21, p. 462, 1926.
5. **Savage.** Sc. Agr. Ottawa, Vol. 4, 1924.
6. **Wodsedalek.** BIOL. BULL., Vol. 25, 1913, 27; 1914, 30; 1916, 38, 1920.
7. **Wodsedalek.** Sc., N. S., Vol. 38, 1913.
8. **Zeleny and Faust.** J. Exp. Zool., Vol. 18, 1915.
9. **Parkes.** Quart. J. Micros. Sc., Vol. 67, 1923.
10. **Hance.** J. Morph., Vol. 30, 1917.
11. **Lush.** Jour. Agri. Res., Vol. 10, 1925.

STUDIES ON THE PANCREATIC SECRETION IN SKATES.

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From the point of view of comparative physiology the skate possesses many features of interest. Animals such as the elasmobranch fishes, which are generally considered to have remained at a lower point of evolution than mammals, present an opportunity of investigating the intermediate stages of functional development of the different organs of the higher forms. An attempt was made in the present study to investigate the pancreatic secretion in skates, since the anatomical relation of the pancreatic gland in these animals affords certain advantages for such experimental study. In addition some observations were made on the distribution and relations of the pancreatic ducts which present certain peculiarities in this animal form.

Anatomical Data.

The pancreatic gland was investigated in three species of skates, namely, *Raja erinacea*, *Raja diaphanes* and *Raja stabuliforis*. The size of the organ varies of course according to the proportions of the animal. In all these the gland is quite compact, showing no tendency to take the diffuse form seen in most of the higher orders of fishes but rather resembling the analogous structure in the higher vertebrates, including man. Indeed the gland is much more compact in the skate than in the rodent type of mammalians. Disregarding minor differences in the shape of the pancreas in the three species examined, one finds that it always consists of two lobes of unequal size connected by a more or less constricted isthmus of pancreatic tissue. The ventral lobe, which is proximal to the intestine, is considerably smaller and lies attached to the groove formed by the junction of the duodenum and the pyloric end of the stomach. The dorsal lobe is much larger and lies under the stomach. In the excised pancreas it flattens to a certain degree (Fig. 1).

For studying the distribution of the duct system, the ducts were in some cases injected with a mixture of carmin and starch in 10 per cent. formalin, and the fixed preparation was then carefully dissected out. In other cases the ducts were injected with pyroxylin dissolved in acetone and coloured with ultramarine-blue or vermillion. A cast of the duct system was then obtained by digesting away the surrounding glandular tissue by means of a mixture of pepsin and hydrochloric acid. At the Biological Station the mixture was made up by adding a quantity of 0.36 per cent. hydrochloric acid to the gastric mucous membrane excised from skates.

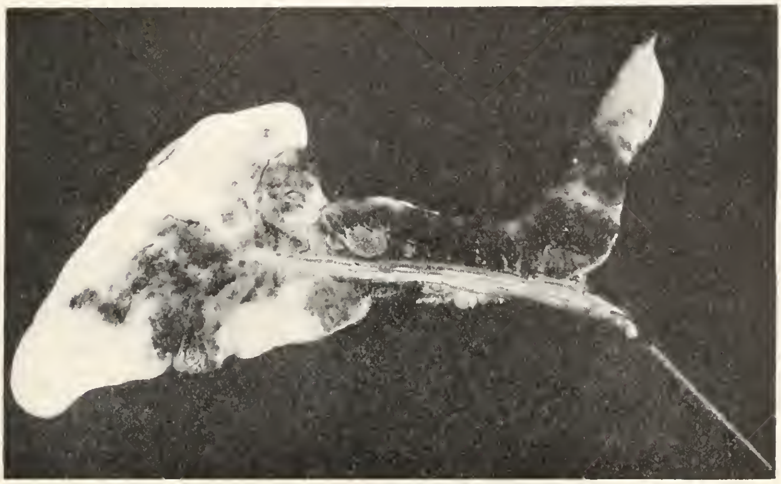

FIG. 1. Pancreatic gland of *R. stabuliforis* injected with carmin-starch-formalin mixture.

There is usually only one duct connecting the gland with the duodenum. This duct takes the form of a comparatively large tube of approximately uniform caliber, from which smaller ducts are given off. The large duct traverses the dorsal side of the isthmus of the gland and disappears into the midst of the distal lobe. Fig. 1 is a photograph of the pancreatic gland of *Raja stabuliforis* seen from the dorsal side and showing the main duct injected. The length of the fish was 121 cm. The length of the visible portion of the main duct was 5.8 cm., while the portion connecting the proximal lobe with the duodenum measured 1.8 cm. The diameter of the visible portion of the duct was about 3.5 mm., but it became slightly reduced toward the point where the duct disappeared into the substance of the distal lobe of the gland. Figs. 2*a* and 2*b* are sketches of the pancreatic ducts of two specimens of *Raja stabuliforis* as they appeared after being injected with the mixture of carmin, starch and formalin. Fig. 3 shows a

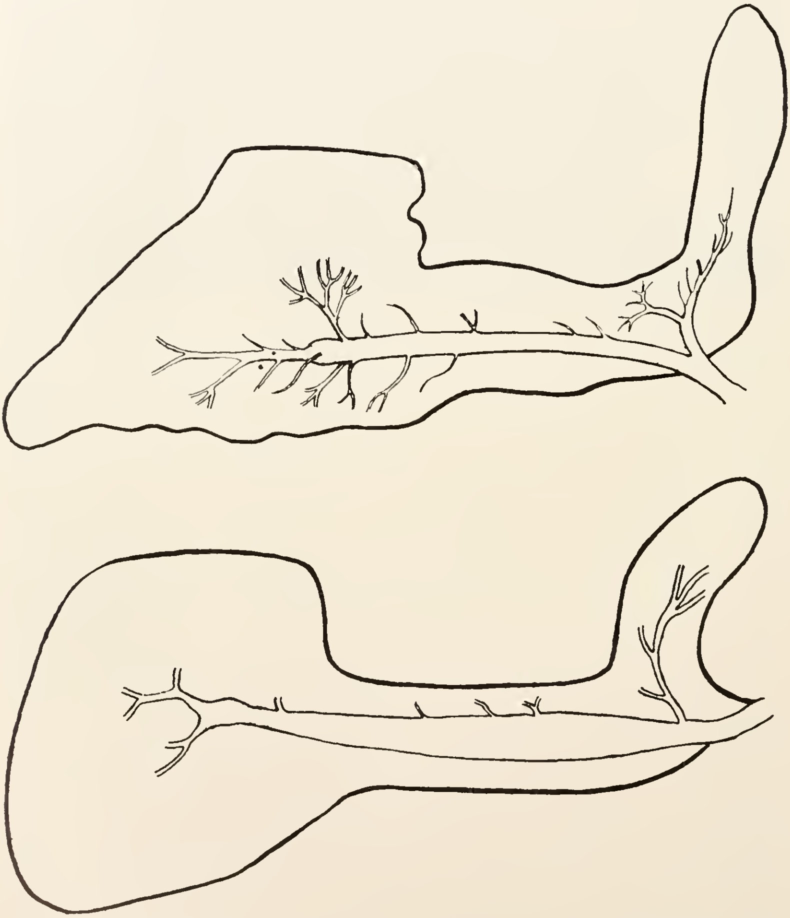

Fig. 2, *a* and *b*. Sketch of pancreatic ducts of two *R. stabuliforis* injected with carmin-starch-formalin mixture. Only a few secondary, tertiary, etc. ducts could be injected. In case "*a*" the main duct presented a large tube of uniform size. In case "*b*" the main duct was dilated in the middle part.

similar preparation of *Raja diaphanes* and demonstrates the analogous structure of the gland in these two species.

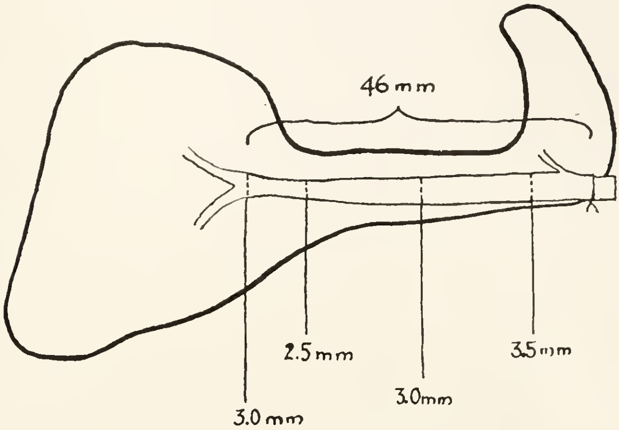

FIG. 3. Sketch of the pancreatic ducts in *R. diaphanes*.

The most striking feature of the duct system in the pancreas of the skate family is the contrast between the calibre of the main duct and that of the secondary ducts. In *Raja stabuliforis*, for instance, the only branch having a fairly large diameter is that which drains the proximal lobe of the gland. All other branches in this species have such extremely small channels that only the very beginning of each could be injected. In *Raja diaphanes* it was found to be very difficult to inject any of the secondary ducts, while in the case of *Raja erinacea* attempts to inject these ducts were entirely unsuccessful.

This peculiar contrast in calibre between the main and the secondary ducts suggests that the latter do not develop as fully in the skate as in higher forms. In mammals we find a gradual diminution in caliber, proceeding from the main duct or ducts through successively smaller divisions until the terminal ductules are reached. In the cat and dog, for instance, even the terminal ductules are of such a caliber that it is comparatively easy to inject the entire system of ducts, as Revell (1) has shown for the pancreas of the dog. On injecting the pancreas of the cat, the writer has observed very thorough penetration of the injection mass into the smallest ductules, but a similar injection could not be made to penetrate the secondary ducts of skates to any extent, or when it penetrated the cast was so fine that the ducts could not be preserved intact during digestion of the pancreatic tissue.

It is interesting to note that in skates the "Langerhans cells" do not form the regular islands of Langerhans as in mammals. According to Jackson (2), most of the "Langerhans cells" in the pancreatic gland of skates remain in contact with the ducts. They are usually found between the cells forming the outer layer of the ducts. Jackson looks on this peculiar distribution of "Langerhans cells" in the skate's pancreas "not as constituting a fundamental difference as compared with other groups (*e.g.* mammals) but as a more primitive condition, of phylogenetic and ontogenetic interest."

It seems that the whole structure of the pancreas has a more primitive character in skates than in mammals. We are now engaged on a special histological investigation of the pancreatic ducts in the skate. It may be added that the pancreatic ducts are also extremely narrow in some bony fishes, and it is almost impossible to inject them, (Legouis (3), Krüger (4)).

METHODS.

Two methods of immobilizing the animals were employed in the present investigation, namely, section of the spinal cord below the medulla and intraperitoneal injection of Dial "Ciba." The first method was based on the investigations of Miss Craw (5), who showed that spinal skates can live for a long time under proper conditions. The operation was performed as follows: A specimen was removed from the water, and its spinal cord was cut quickly below the medulla. A glass tube connected with the sea-water pipe system was inserted into one of the spiracles. If the section of the spinal cord was performed not too near the medulla oblongata respiratory movement continued for several hours. After section of the spinal cord the animal was turned on its back and the abdomen opened. A cannula connected with a graduated tube was then inserted into the pancreatic duct. The common bile duct was ligatured near the duodenum and a cannula connected with a graduated tube was fixed into the gall bladder. Through an opening in the pyloric part of the stomach a glass cannula (for injections into the duodenum) was inserted into the duodenum and tied. In some of the experiments, to prevent filling of the stomach with sea water, which runs through the spiracles and is sometimes swallowed by the animal, the œsophagus was tied near the cardia. In many of the experiments the rectum was also tied to prevent the escape of the solutions introduced into the duodenum.

In the case of immobilization by "Dial" Ciba (Basel) the animal was taken from the water, placed on its back and held by a wire-net with loops big enough to pass through a hypodermic needle. An injection was then made into the abdominal cavity by means of a syringe with "Dial" 0.35 to 0.40 c. cm. per kilo weight, and the animal put back in the water. In about a quarter of an hour the animal was asleep and underwent the operation described above. The dose of Dial required was much smaller than the corresponding dose for warm-blooded animals (*e.g.* 0.7 c.cm. per kilo weight for a dog or cat). Notwithstanding the skates were perfectly anæsthetized for 24 hours or more with this amount of Dial. Larger doses of Dial stopped the breathing, though it could usually be restored by means of artificial respiration.

The reaction of the pancreatic juice, bile and gastric contents was tested with litmus paper, and whenever possible the hydrogen ion concentration was determined by the colorimetric method.

The proteolytic activity of the pancreatic juice was determined by means of digestion of fresh calf's fibrin, preserved in glycerin and washed thoroughly before the experiment with running water. Protrypsin was activated by an extract of the intestinal mucous membrane of the skate.

The diastatic power of the juice was tested with 1 per cent. soluble starch solution (iodine and Fehling's tests).

The lipolytic power of the juice was determined according to the method of Anrep, Lush and Palmer (6).

The methods of preparation of the different extracts for enzyme determinations will be described later.

ELASTICITY AND CONTRACTILITY OF THE MAIN PANCREATIC DUCT.

Before presenting data concerning the pancreatic secretion the properties of elasticity and contractility of the main pancreatic duct will be discussed. These properties of the duct determined some special measures employed during the collection of the pancreatic juice. It could be seen very often in the course of an experiment that the fluid in the graduated tube instead of moving forward moved backwards a few divisions. Slight massage of the duct propelled the juice along the tube.

This backward movement of the fluid may sometimes be observed at the beginning of an experiment in spinal animals in quite good condition, but it is seen very often at the end of an experiment when the animal is dying or even shortly after death. Cutting the spinal cord too close to the medulla, which is usually followed by difficulty in respiration, has the same effect. In skates anæsthetized with Dial the contraction of the duct was not at all marked. Thus the main duct possesses a tone of its own, which may be increased or diminished under certain conditions. That we are not dealing with mere elasticity of the walls is shown by the following experiment:

Exp. Aug. 12. R. diaphanes. Spinal preparation. The pancreatic cannula and the graduated tube were filled with filtered sea water containing 0.5 per cent. of urea. The freezing point of this fluid was equal to $-1.89°$ C. The $\triangle$ of the blood of *R. diaphanes* is equal to $-1.80°$ C. (For these determinations I am indebted to Mr. A. F. Chaisson, who worked at the St. Andrews Biological Station.) This fluid was an indifferent one for the tissue of the skate.

From 10 A.M. to 10:30 A.M. the fluid moved from division 139 to division 140 of the graduated tube (1 division). At 10:30 A.M. the graduated tube was turned upright, and the level of the fluid sank very rapidly to 107 (33 divisions). The graduated tube was then closed and the rubber tube connecting it with the pancreatic cannula was twice gently compressed. As a result of this more fluid entered the gland, so that the fluid in the cannula moved up 36 divisions and now stood at 71. When the graduated tube was opened the fluid rose in it to 79 (8 divisions) and in five minutes fell again to 75 (4 divisions). Fifteen minutes later the level was at 74, and some fifteen minutes after that at 73. When the tube was placed horizontally, the fluid moved along it 25 divisions *i.e.*, reached the 98th division.

It may be seen from this experiment that sudden distention of the duct stimulated it to contract. Later it relaxed. When the pressure on the walls of the graduated tube was diminished by placing it horizontally, the fluid moved along 25 divisions. This phenomenon must be ascribed to the elasticity of the duct. Some of the fluid pressed into the gland did not return, being absorbed, or as seems more probable, remaining in the small ducts or in the interstitial tissue.

The preliminary histological investigation of the pancreatic gland in skates, performed in our laboratory by Dr. D. J. Bowie, showed that in the vicinity of the main pancreatic duct there are smooth muscular fibers.

In connection with these findings a method of very gentle pressure on the main duct during the secretory periods was adopted. This ensured that all pancreatic juice secreted during a certain time passed into the cannula and the graduated tube.

Pancreatic Secretion.

A spontaneous pancreatic secretion was noticed in almost every case. In the experiments the secretion in animals with an empty stomach and duodenum was very scanty. In *R. diaphanes* it averaged only 0.02 c.cm. in one hour. Sullivan (7) has attempted to collect the pancreatic juice in *Carcharias littoralis* over several days. Through an incision in the abdomen a glass cannula was inserted and fastened in the central end of the pancreatic duct. To the outer end of the cannula a small sterilized balloon was attached. Although there was great difficulty in keeping such fish alive, the operation was successful in six cases. The quantity of juice thus collected by Sullivan was as a rule small, however, and it had no digestive activity.

The narrowness and fragility of the pancreatic duct in *Scyllium catulus* and *Lamna cornubica* according to Yung (8) make the preparation of a fistula in the living animals impossible.

Attempts were made to stimulate pancreatic secretion by the following methods: (1) Introduction into the duodenum of HCl solution of different concentrations (0.36 per cent. to 0.95 per cent.). (2) Introduction of a mixture of equal parts of 0.36 per cent. HCl + 2 per cent. urea solution. (3) Intravenous injection of secretin (a cannula was inserted in the central end of one of the gastric veins; secretin was prepared in the usual manner by the action of 0.36 per cent. HCl on the mucous membrane of the duodenum and of the spiral intestine of the skate). (4) Injection of pilocarpin hydrochloride solution intravascularly and into the ducts of the pancreas.

The introduction of HCl solution into the duodenum usually had a definite positive secretory effect. 0.49 per cent. HCl solution was more effective than 0.36 per cent. and 0.96 per cent. solutions.

In some cases the action of the hydrochloric acid was very insignificant. This was usually the case in fasting animals. On the other hand, the introduction of an acidified peptone solution or of an acid digest of fish muscle increased the subsequent secretory effects of HCl solution. In the experiment quoted below (Experiment of July 23) 0.49 per cent. HCl injected into the duodenum after an acid digest, gave the greatest secretion of pancreatic juice ever observed in these experiments.

Exp. July 23. R. diaphanes. ♂. Weight 4416g. 1.5 c. cm. Dial injected at 8:15 A.M. Operation from 8:40 A.M. to 9 A.M. Stomach contained a small amount of food. Duodenum cannulated. Tying of the pylorus produced vomiting, also observed in several previous experiments. Pancreatic duct cannulated. The secretion was noted in the divisions of the graduated tube every 30 minutes.

Time.	Secretion in Divisions.
9 A.M. to 9:30 A.M.	0.5
9:30 A.M. to 10 A.M.	4.5
10 A.M. to 10:30 A.M.	2.0
10:30 A.M. 50 c. cm. of digest[1] injected into the duodenum	
10:30 A.M. to 11 A.M.	5.5
11 A.M. to 11:30 A.M.	2.5
11:30 A.M. to 12 noon	8.5
12 noon to 12:30 A.M.	7.5
12:30 P.M. to 1 P.M.	7.0
1 P.M. to 1:30 P.M.	10.0
1:30 P.M. to 2 P.M.	
2 P.M. to 2:30 P.M.	4.0
2:30 P.M. 50 c. cm. of digest injected into the duodenum. The fluid was partly evacuated from the anal opening.	
2:30 P.M. to 3 P.M.	3.5
3 P.M. to 3:30 P.M.	3.5
3:30 P.M. to 4 P.M.	3.0
4 P.M. to 4:30 P.M.	3.0
4:30 P.M. Rectum tied. 50 c. cm. of 0.49 per cent. HCl injected into the duodenum.	
4:30 P.M. to 5 P.M.	3.0
5 P.M. to 5:30 P.M.	12.0
6 P.M. to 6:30 P.M.	13.0
6:30 P.M. to 7 P.M.	11.0
7 P.M. to 7:30 P.M.	16.0
7:30 P.M. to 8 P.M.	
8 P.M. to 8:30 P.M.	10.0
8:30 P.M. to 9 P.M.	7.0

[1] The digest was prepared as follows: Two stomachs of skates and skate's muscles were mixed July 22 with 0.47 per cent. HCl solution and placed in the incubator (10 A.M.). July 23 (9 A.M.) almost all was digested. The fluid acquired a brownish colour. pH of this fluid was 4.2.

Experiment stopped. Animal was in good condition. Amount of pancreatic juice secreted during 12 hours 0.55 c. cm. Its pH = 7.2. Weight of the pancreatic gland 5.35 g. Content of the duodenum alkaline on litmus. Stomach contained water and mucus only, reaction slightly acid on litmus.

The last part of the experiment, *i.e.*, the action of 0.49 per cent. HCl solution, is represented graphically in Fig. 4. The quantities of juice secreted are shown in actual volumes in c.cm. The curve is typical for the action of hydrochloric acid. It is reminiscent of the corresponding curve in warm-blooded animals (dog, man), which generally shows a sharp rise before reaching its peak, and then gradually descends. There is however a stricking difference in the time required for this phenomenon in warm-blooded animals and in the skate. Thus the latent period for the secretory action of HCl in dogs is from $1\frac{1}{2}$ minutes (dogs with a permanent pancreatic fistula) to 4–5 minutes (acute experiments). In skates it requires half-an-hour for the acid to develop its secretory effect. Secretion on 200 c.cm. of 0.5 per cent. HCl in a

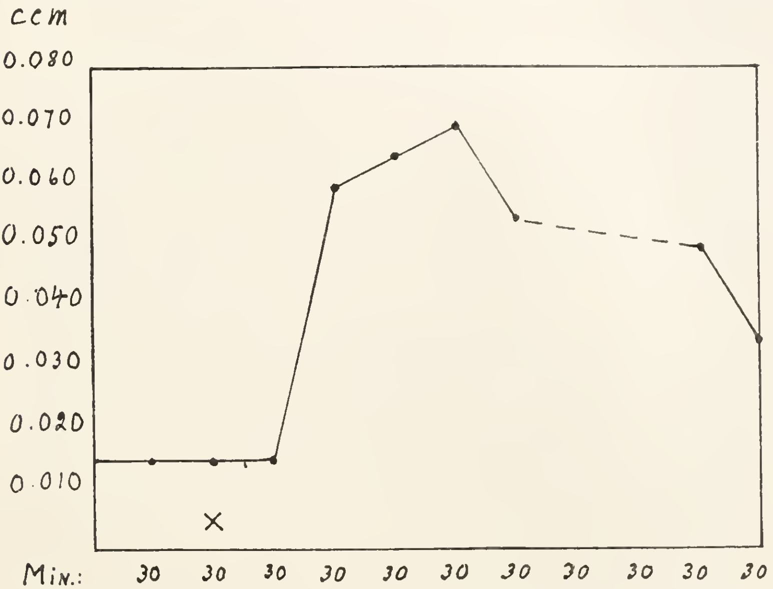

Fig. 4. Curve of the pancreatic secretion in a skate after injection of 0.49 per cent. solution of hydrochloric acid. The ordinates represent the amount of pancreatic juice in c. cm. Every division of abscissa is equal to 30 minutes. At *X* 50 c. cm. of acid was injected into the duodenum.

dog of 15 to 20 kilos with a permanent pancreatic fistula lasts about an hour and a half to two hours, and somewhat longer in an acute experiment. In a skate of 4 to 5 kilos weight the pancreatic secretion on 50 c.cm. of 0.5 per cent. HCl extends over 4 or more hours. Another feature of the pancreatic secretion in skates is its scantiness as compared with the secretion similarly stimulated in dogs. Thus in the experiment on a skate quoted above the amount of pancreatic juice secreted on 50 c.cm. of 0.5 per cent. HCl during four hours was 0.43 c.cm. In a dog of approximately four times greater weight, a correspondingly greater amount of 0.5 per cent. HCl, *i.e.*, 200 c.cm., in 1 hour, and 45 minutes, gave 138.0 c.cm. of juice (Dolinski (9)). One of the factors responsible for the scantiness of the pancreatic secretion in the skate is its low body temperature as compared with warm-blooded animals. Thus the temperature of the water running through the gils in this experiment was 13.5° C. The water in the tank which was exposed to the air of the laboratory was 15° C., and the temperature of the fish was probably about the same. If Van't Hoff's law could be applied to the secretory processes, namely, that the velocity of a chemical reaction is doubled with each 10° C. rise of temperature, it still could not explain the difference in the activity of the pancreatic gland in warm- and cold-blooded animals. The most probable explanation of the scarcity of the pancreatic secretion in skates is that this organ is not developed to the same degree as in warm-blooded animals. Thus, in dogs, for example, the average weight of the pancreatic gland is approximately $\frac{1}{400}$ of the body weight, whereas in *R. diaphanes* it averages only $\frac{1}{800}$ of the body weight.

No essential difference was noted in the secretory action of the hydrochloric acid alone or when mixed with 2 per cent. urea, one of the permanent constituents of the blood of this fish.

The humoral character of the pancreatic secretion in the skate was emphasized by the secretory action of secretine prepared on 0.36 per cent. hydrochloric acid. The following experiment is quoted as an example:

Exp. July 26. Raja diaphanes. Spinal preparation. Stomach empty. Pancreatic duct and common bile duct cannulated. Secretion noted every 15 min. in divisions of graduated tubing.

Time.	Secretion.
3:25 P.M. to 3:40 P.M	3.5
3:40 P.M. 12:05 c. cm. of secretine injected into one of the gastric veins.	
3:40 P.M. to 3:55 P.M	2.5
3:55 P.M. to 4:10 P.M	13.0
4:10 P.M. to 4:25 P.M	2.0
4:25 P.M. to 4:40 P.M	3.0
4:40 P.M. to 4:55 P.M	3
4:55 P.M. to 5:10 P.M	0

Even after direct introduction into the blood of the secretory agent, *i.e.*, of the secretine, the latent period of secretion was equal to 15 minutes. This fact may probably ve explained by the slowness of the circulation in skates. In the summer time, under the conditions of the experiment, the heart usually contracted only 22 to 26 times in a minute.

The data reported above show that hydrochloric acid in a concentration of about 0.5 per cent. stimulates the pancreatic secretion in skates. The action of the hydrochloric acid is greater after previous introduction into the duodenum of the acid products of protein digestion. The mechanism of the secretory action of HCl is probably humoral, through the formation of secretine.

Experiments with Pilocarpin.

Different kinds of experiments were devised to ascertain the effect produced by pilocarpin on the secretory function of the pancreatic gland, using it as a drug to stimulate the peripheral parts of the parasympathetic nervous system. The following procedures were adopted: (1) Intravenous injection of 0.1 per cent. pilocarpin hydrochloride solution in variable amounts (from 2 to 4 mg. or more). The solution was usually injected with a fine hypodermic needle into one of the gastric veins or into the portal vein itself. (2) Injection of the pilocarpin solution into the conus arteriosus or into the ventricle of the heart. (3) Injection of the solution into the superior mesenteric artery, which supplies with blood the whole dorsal part and half the isthmus of the pancreatic gland, *i.e.* the greater part of the organ. (In several experiments the pilocarpin solution was stained with methylene blue.) (4) Introduction of the pilocarpin solution into the pancreatic ducts. For this purpose the graduated tube and the cannula were filled with the solution, and kept in a vertical position for 15 minutes. Under its own pressure the solution entered the duct and distended it. The graduated tube was then returned to the horizontal, and the rate of secretion noted.

In no case did pilocarpin activate the pancreatic secretion or increase spontaneous secretion. Though the negative results in some of the experiments might be explained by the damming back of the pilocarpin solution owing to the narrowness of the small pancreatic ducts, through which the solution could not penetrate to the alveoli, an analogous explanation could not be applied to the experiments where the drug was injected into the arterial system.

As an example of the action of pilocarpin the Experiment of July 30 is quoted.

Exp. July 30. R. diaphanes ♀ Weight 5010 g. 8:15 A.M. Dial injected intraperitoneally. 8:30 to 8:50 A.M. abdomen opened; pancreatic duct cannulated. There was spontaneous pancreatic secretion, which became very insignificant about 1:15 P.M. Skin and muscles covering the heart chamber removed. Pancreatic secretion was noted in divisions of graduated tubing every 15 min. unless marked otherwise.

Time.	Secretion.	Heart Rate Beats per Minute.
1:15 P.M. to 1:30 P.M.	2	
1:30 P.M. to 1:45 P.M.	1	
1:45 P.M. to 2:00 P.M.	0	22
2 P.M. 2 mg. of pilocarpin hydrochloride in 2 c. cm. of distilled water injected into the bulbus aortae. 2.03 P.M. heart 12 beats per minute; 2.06 P.M. heart 18 beats per minute.		
2:00 P.M. to 2:15 P.M.	1	18
2:15 P.M. to 2:30 P.M.	1	18
2:30 P.M. to 2:45 P.M.	1	18
2:45 P.M. to 3:00 P.M.	1	
3 P.M. 2 mg. pilocarpin hydrochloride in 2 c. cm. of distilled water injected into the bulbus aorta. 3.05 P.M. heart 18 beats per minute.		
3:00 P.M. to 3:15 P.M.	0	16
3:15 P.M. to 3:30 P.M.	0	16

3:35 P.M.	4 mg. of pilocarpin hydrochloride in 4 c. cm. of methylene-blue solution injected into the superior mesenteric artery. The main lobe of the pancreatic gland and half the isthmus became blue. Heart rate at 3.40 P.M. 8 per min. Respiratory movements stopped and did not recover till the end of the experiment. Sea water continued to run through the gills. 3:47 P.M. heart rate 14 per min.		
3:35 P.M. to 4:00 P.M.		1	16
4:00 P.M. to 4:15 P.M.		0	8

This experiment shows that pilocarpin introduced into the arterial system in no way stimulates the pancreatic secretion. The heart rate is influenced by pilocarpin, though in far lesser degree than in warm-blooded animals.

Properties of the Pancreatic Secretion.

Although the pancreatic secretion in skates was so scanty (especially in *R. erinacea*), a certain amount of juice (0.2 to 0.5 c.cm.) was obtained in almost every experiment. Pancreatic juice was also collected from the main duct of freshly caught *R. stabuliforis* (in some cases to the amount of 0.5 c.cm.).

The pancreatic juice of the three species investigated is a colourless, almost neutral fluid. The hydrogen ion concentration of the juice determined colorimetrically (Felton's (10) spot method or British Drug Houses Capillator) varied from 6.6 to 7.2 (eleven determinations).

For the determination of its enzymatic activity, the pancreatic juice was usually diluted with distilled water, and the hydrogen ion concentration of the mixture was adjusted to a certain point by means of anhydrous sodium carbonate or corresponding buffer solutions.

These experiments showed that the pancreatic juice of the skate possesses proteolytic, diastatic and lipolytic action. The proteolytic action was increased by adding an extract of the intestinal mucous membrane to the pancreatic juice. The diastatic ferment was effective without any activator. In what form, *i.e.* active or inactive, the pancreatic lipase is secreted one cannot say since in the method of determination of Anrep, Lush and Palmer (6) sodium glycocholate is used, and this activates the prolipase.

Thus the pancreatic juice of skates contains all three enzymes which are found in the pancreatic juice of higher mammals including man.

To prove conclusively that the pancreatic gland of skates produces these enzymes several experiments were performed with pancreatic extracts. This was the more important since Yung (8) reported that some of the pancreatic extracts of *Scyllium catulus* and *Lamna cornubica* were inactive towards fibrin but always active in the digestion of starch and emulsification of fat. Sullivan (7) could not demonstrate any amylolytic action of water-glycerin extracts of the pancreatic gland of *R. erinacea.*

The pancretic extracts were prepared on 30 per cent ethyl alcohol, the extracts of the intestinal mucous membrane with 0.9 per cent NaCl. They were kept with toluene for several days at room temperature and then filtered through cheese-cloth.

As an example I quote one of the experiments with *R. erinacea.*

Exp. July 22. R. erinacea. The pancreatic gland (weight 1 g.) extracted for three days with 2 c. cm. of 30 per cent. alcohol. July 25, filtered through cheese-cloth. Mucous membrane of the duodenum and of the spiral valve extracted with 0.9 per cent. NaCl for three days.

PANCREATIC AMYLASE.

July 25.	*Control.*
3:50 P.M. 3 drops of pancreatic extract plus 7 drops of 1 per cent soluble starch solution, plus 7 drops of distilled water, plus one drop of toluene, pH = 6.6, in incubator at 37° C.	3:50 P.M. 7 drops of 1 per cent. soluble starch solution plus 7 drops of distilled water, plus one drop of toluene, pH = 6.6, in incubator at 37° C.
7:00 P.M. Reaction with iodine—colorless.	7.00 P.M. Reaction with iodine—blue.
8:00 P.M. Ditto. Fehling distinctly positive.	8:00 P.M. Ditto. Fehling negative.

PANCREATIC LIPASE.

July 26.	*Control,*
11:00 A.M. 3 drops of pancreatic extract plus 2 c. cm. of buffer solution, pH = 8.0, plus 2 c. cm. of glyceroltriacetate, plus 9 drops of sodium glycocholate solution, plus 6 drops of phenol red, plus toluene.	11:00 A.M. Everything in same proportion, except pancreatic extract which was not added.
8:00 P.M. Still pink.	8:00 P.M. Pink.
July 27.	
8:00 A.M. Yellow (pH = 7.0).	8:00 A.M. Pink.

Pancreatic Protease.

July 27.		Control.
10:15 A.M. 5 drops of pancreatic extract, plus 5 drops of distilled water, plus one drop of phenol red, plus one drop of toluene, pH adjusted with Na_2CO_3 to 8.0 and fibrin added.	10:15 A.M. Ditto, plus one drop of intestinal extract.	10:15 A.M. 5 drops of intestinal extract, plus 5 drops of distilled water, plus one drop of toluene, plus fibrin. pH = 8.0.
12:30 P.M. No change.	12:30 P.M. Completely digested.	12:30 P.M. No change.
8:00 P.M. No change.		8:00 P.M. No change.
July 28.		
10:15 A.M. No change. Added one drop of intestinal extract.		10:15 A.M. No change. Added 5 drops of pancreatic extract.
1:00 P.M. Completely digested.		1:30 P.M. Completely digested.

This experiment shows that the pancreatic gland of *R. erinacea* possesses diastatic, lipolytic and proteolytic action. The pancreatic protease is contained in the gland in the form of zymogen.

Experiments with the alcoholic extracts of the pancreatic gland of *R. diaphanes* gave similar results. There were some differences in the rapidity with which the pancreatic enzymes of *R. diaphanes* acted when compared with the action of corresponding enzymes of *R. erinacea*. Thus in the Experiment of August 2, the alcoholic extract of the pancreas of *R. diaphanes* changed the reaction of glycerol-triacetate mixture from pH = 8.0 at 9:10 A.M. to pH = 7.4 at 3:30 P.M., pH = 7.2 at 6:00P.M., and finally to pH = 7.0 at 7:30 P.M. Since the enzymatic strength of extracts prepared from one and the same species of fish varied in different extracts, these variations are to be attributed more to the mode of extracting than to real difference in the content of enzymes in the gland.

One point is worth mentioning. According to Sullivan (7) the water-glycerin extracts of pancreas of different elasmobranch fishes did not digest either the coagulated protein of Mett's tubes or fibrin. These extracts digested gelatine only after activation with water-glycerin extract or chloroform extract of the duodenal mucous membrane, or the extract of the mucous membrane of the spiral valve, the last being the most effective.

As may be seen from this study all pancreatic extracts of *R. erinacea* and *R. diaphanes*, as well as the pancreatic juice of these two species and of *R. stabuliforis*, after activation with the intestinal extract, digested fibrin rapidly.

To verify Sullivan's statement that the mucous membrane of the spiral valve contains more enterokinase than that of the duodenum, special experiments were performed. Samples of the same pancreatic extract *R. diaphanes* were activated with 0.9 per cent NaCl extracts of the mucous membrane of the duodenum and the spiral valve (also *R. diaphanes*). The results were as follows:

The pancreatic extract 15 drops (diluted twice with water and with pH adjusted to 8.0) did not digest fibrin in 48 hours.

The same pancreatic extract in the same dilution with the addition of 4 drops of duodenal mucous membrane extract, digested fibrin in 11 hours.

The same pancreatic extract in the same dilution, plus 4 drops of spiral valve extract, digested the same amount of fibrin in about 20 hours.

Both intestinal extracts alone were inactive towards fibrin.

Thus the duodenal extract showed greater activating power than the spiral valve extract. This was probably due to the presence of a greater amount of mucus in the latter.

Note on the Secretion of Bile

The special arrangement of the experiments in this investigation (tying of the common bile duct and insertion of a cannula into the gall bladder) made it possible to study the bile secretion. The bile (usually dark or emerald green gall-bladder bile in fasting animals) was pressed out from the viscus. The freshly secreted bile was of a straw-yellow color. The reaction of the gall-bladder bile in *R. diaphanes* was slightly acid (average pH = 6.4). The reaction of the gall bladder bile in *R. erinacea* according to Miss Mackay (11) was in average pH 6.3. The reaction of the hepatic bile was slightly alkaline (pH 7.5 to 7.6). The secretion of bile was slow and scanty, although more copious than that of the pancreatic juice. The average rate of bile secretion in fasting *R. diaphanes*, without the application of any stimuli, was from 0.01 to 0.02 c.cm. in thirty minutes. In a successful long experiment more than 1 c.cm. of freshly secreted bile was obtained. In *R. erinacea* the secretion was much slower, producing on the average 0.01 c.cm. per hour.

Introduction into the duodenum of 0.36 per cent. to 0.49 per cent. HCl solutions, in some cases mixed with bile, as well as 10 per cent. Witte's peptone solution, increased the secretion of bile, sometimes doubling it.

CONCLUSIONS.

Although the pancreatic gland of the skate secretes the same enzymes as the pancreas of mammals, it seems that in the skate this organ has not attained to the high stage of development of the mammalian pancreas. This is indicated by the scantiness of the pancreatic secretion in skates, the peculiar arrangement of the secondary pancreatic ducts, marked by their narrowness, and the smaller weight of the pancreatic gland in relation to the body weight as compared with warm-blooded animals.

Under the experimental conditions described the pancreatic secretion in skates is continuous but scanty. The hydrogen ion concentration of the pancreatic juice is equal to pH 6.6 to 7.2. This again is a special feature of the secretion, since the pancreatic juice in mammals (dog, cat, man) is decidely alkaline (average pH = 8.4). Whereas in mammals the pancreatic juice plays an important part in the neutralisation of acid chyme entering the duodenum, in skates the scanty and almost neutral pancreatic secretion cannot be an important factor in this respect. The reaction of the gall-bladder bile in skates is slightly on the acid side, and that of the hepatic bile very faintly alkaline. Nevertheless the reaction in the duodenum is strongly alkaline, this being evidently due to the alkaline secretion of the succus entericus

Hydrochloric acid introduced into the duodenum activates the pancreatic secretion, probably in a humoral way, since intravenous injections of secretine produce a positive secretory effect. Parasympathetic poison, such as pilocarpin, does not influence the pancreatic secretion in any way.

SUMMARY.

1. The system of pancreatic ducts in *R. erinacea, diaphanes* and *stabuliforis* presents certain peculiarities which differentiate it from the analogous system of ducts in higher mammalian animals.

2. Pancreatic secretion in skates which have previously fasted

for several days is continuous but very scanty. Hydrochloric acid and secretin increased this secretion. Pilocarpin was without effect. The previous introduction into the duodenum of the acid digest of proteins increased the secretory effect of the hydrochloric acid.

3. The pancreatic juice is a neutral fluid (pH = 6.6 to 7.2), possessing proteolytic, diastatic and lipolytic action.

4. Alcoholic extracts of the pancreas show the same enzyme action as the juice. A proteolytic enzyme is contained in the gland in the form of protrypsin, which may be activated by 0.9 per cent. NaCl extract of the mucous membrane of the duodenum and spiral valve.

5. Bile is secreted continuously. Introduction of 0.36 per cent. HCl solution and 10 per cent. Witte's peptone solution into the duodenum increases the secretion.

The author wishes to acknowledge his indebtedness to the Biological Board of Canada for permission to use the facilities of the Atlantic Biological Station, St. Andrews, N. B., and to Professor A. G. Huntsman and his staff for valuable assistance rendered in the course of this study.

REFERENCES.

1. **D. G. Revell.**
   '02 The Pancreatic Ducts in the Dog. Amer. Journ. of Anat., Vol. I., 443.
2. **S. Jackson.**
   '23 The Islands of Langerhans in Elasmobranch and Teleostean Fishes. Journ. of Metabol. Research, Vol. II., 2.
3. **P. Legouis.**
   '73 Recherches sur les tubes de Weber et sur le pancréas des poissons osseux. Annales des Sci. Nat. Zool., T. 17, 18.
4. **A. Krüger.**
   '05 Untersuchungen über das Pankreas der Knochenfische. Wissenschaft. Meeruntersuchungen. Abt. Kiel. N. F., Vol. 8, 57.
5. **H. Craw.**
   '27 The Spinal Reflexes of the Skate. Journ. of Physiol., Vol. 63, 61.
6. **G. V. Anrep, J. L. Lush and M. G. Palmer.**
   '24-'25 Observations on Pancreatic Secretion. Journ. of Physiol., Vol. 59, 434.
7. **M. X. Sullivan.**
   '07 The Physiology of the Digestive Tract of Elasmobranchs. Bull. of the Bureau of Fisheries, Vol. 27, 1. 1907. See also Amer. J. Physiol., Vol. 15, 42. 1905–06.
8. **E. Yung.**
   '98 Sur les fonctions du pancreás chez les squales. C. R. de l'Acad. des Sci., Vol. 127, 77.

9. **J. L. Dolinski.**

'94 Diss. St. Petersburg, 1894, quoted by B. P. Babkin: Die äussere Sekretion der Verdauungsdrüsen 2d ed., P. 501. Berlin, 1928.

10. **L. D. Felton.**

'21 A Colorimetric Method for Determining the Hydrogen Ion Concentration of Small Amounts of Fluid. Journ. Biol. Chem., Vol. 46, 299.

11. **M. E. Mackay.**

'29 Note on the Bile in Different Fishes. BIOL. BULL., Vol. 56, 24.

THE RESISTANCE OF THE FRESHWATER SNAIL, *PHYSA HETEROSTROPHA* (SAY) TO SEA WATER.

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The freshwater snail *Physa heterostropha* has been observed in the brackish water of Barnegat Bay near the mouth of the Metedeconk River, Bay Head, N. J. An interesting problem presented itself; how far into brackish water can these freshwater snails migrate? To test this some preliminary experiments were attempted in the summer of 1928. By gradually increasing the salinity of the water *Lymnæa stagnalis appressa* and *L. palustris* (from Michigan) were made to live in 25 per cent. sea water. *Physa heterostropha* (from Philadelphia) died soon after being placed in 10 per cent. sea water. This stock was probably weak. These experiments were summed up in a preliminary report (Richards, 1929).

The summer experiments were of a very preliminary nature and rather crude; so additional and more accurate experiments were begun in the fall of the same year. The work, which was concentrated upon the one species, *P. heterostropha*, seemed to divide itself into two parts: first the experiments dealing with the ability of the species as a whole to withstand gradual and sudden increases in the concentration of sea water; and second, the reactions of the various stocks of the same species to the salt water.

The writer wishes to express his thanks to Dr. H. Burrington Baker and Dr. Edward D. Crabb, both of the University of Pennsylvania, for suggestions and valuable information as to the proper food for the snails and for help in other ways.

METHODS AND PROCEDURE.

In order to determine whether there were any racial differences between various lots of the same species, several sets of controls were kept.

Control.	Locality.	Date collected.
A	Schuylkill River, Fairmount Park, Philadelphia, Pa.	Oct. 16, 1928
B	Schuylkill River, Fairmount Park, Philadelphia, Pa.	Oct. 27, 1928
C	Branch of Cobbs Creek, Haverford, Pa.	Oct, 28, 1928
D	Davies Lake, Cape May Point, N. J.	Nov. 11, 1928
E	Davies Lake, Cape May Point, N. J.	Mar. 30, 1929
F	Wissahickon Creek, Fort Washington, Pa.	Apr. 6, 1929
G	Pond, Botanical Gardens, University of Pennsylvania, Philadelphia, Pa.	Aug. 7, 1929
H	Davies Lake, Cape May Point, N. J.	Aug. 11, 1929

The snails were brought to the Zoölogical Laboratory of the University of Pennsylvania, where they were placed in culture jars. The water used in these experiments was tap water which had been allowed to stand for several days in order to eliminate some of the chlorine so abundant in Philadelphia tap water. In all cases the snails were allowed to live in the fresh water for one week in order to adjust themselves to any possible change. The mortality of the control was found to be highest during the first week.

After the first week some snails were placed in 5 per cent sea water,[1] and a 5 per cent. increase per week was continued until the water reached 30 per cent.; at this point serious effects of the salts were noticed in the behavior of the snails and therefore some were allowed to remain in this concentration with the hope that they might become adjusted to it; others were placed in stronger sea water.

Additional experiments were attempted in which the 5 per cent. increase was made at intervals of two days and one day. Other snails were placed directly in 5 per cent., 10 per cent., 15 per cent., 20 per cent. and 25 per cent. sea water in order to see if they could stand the sudden change.

The sea water used to make up these solutions in all the experiments except part 3 was taken from the vivarium of the University of Pennsylvania. This water had been brought from Ocean City, N. J., some months previous. In the experiments of part 3, which were conducted at Cape May Point, N. J., water from the ocean was used.

The specific gravity of the vivarium water is kept relatively constant at 1.023 (at 17.5°C.). The principal difference between vivarium water and normal sea water was found to be in the hydrogen ion concentration. Normal sea water (off New Jersey) has a pH between 8.1 and 8.3, whereas the water in the tank is more acid, varying from pH 7.8 to 7.9. The acidity is probably due to the acid excrement and dead organic matter from the marine animals in the aquarium.

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[1] Normal sea water being taken as 100 per cent.

The snails were fed green lettuce several times a week, and the old lettuce and excrement removed at frequent intervals, the procedure recommended by Crabb (1929) for the best growth of pond snails.

The temperature was not regulated; it usually lay within the interval between 15° and 20° C.

Results.

Part 1, *5 per cent. Increase in Concentration Every Week.*

Physa lived actively in water as strong as 25 per cent. sea water. Above 25 per cent. the harmful effects of the salts were noted. Above this concentration the activity of the snails was considerably decreased. Snails from control *D* (Cape May Point, N. J.) were considerably more active in 30 per cent. sea water than those from any other control.

Snails were allowed to remain in water of between 25 per cent. and 30 per cent. for several weeks in order to see if they gradually became adjusted to this concentration. However no such adjustment seemed to take place during the several weeks, although throughout the experiments the snails from control *D* were more active in the brackish water than any of the others.

After various intervals the snails were placed in higher concentrations (up to 50 per cent.). In all cases they died, except those in *D*, which were inactive but still alive. Upon being transferred to 5 per cent. sea water they soon revived and were as active as ever.

These experiments are summarized in the following two tables. Table I. deals with the experiments with the 5 per cent. increase in concentration as far as 30 per cent., at which point this method was discontinued.

TABLE I.

SHOWING RESULTS OF EXPERIMENTS IN WHICH THE 5 PER CENT. INCREASE IN CONCENTRATION WAS MADE EVERY WEEK.

The number of snails alive at the beginning and end of each week is given. The summation of all experiments is also given.[1]

		5%	10%	15%	20%	25%	30%
A control	33–19	13–11	11–11	11–11	11–11	11–11	11–11
Experiment		5–5	5–5	5–5	5–5	5–5	5–5
B control	70–67	52–52	52–47	47–45	45–45	40–40	40–40
Experiment		10–10	10–10	10–10	10–10	10–10	10–10
C control	149–139	100–95	95–93	83–83	83–83	83–83	83–83
Experiment		15–15	15–15	15–15	15–15	15–15	15–15
D control	50–50	50–50	50–50	50–50	50–50	50–50	50–50
Experiment		10–10	10–10	10–9	9–9	9–9	9–9
Summation controls	302–275	215–208	208–201	191–189	189–189	184–184	184–184
Experiments		40–40	40–40	40–39	39–39	39–39	39–39
% surviving controls	91.0%	92.3%	96.6%	98.9%	100%	100%	100%
Experiments		100%	100%	97.5%	100%	100%	100%

Table II. surveys the experiments in water of higher concentration than 30 per cent. Since the procedure following the period of attempted adjustment differed in the various experiments, and would be difficult to represent in strictly tabular form, the procedure and results are summarized in a few words in Table II.

Part 2, *5 Per Cent. Increase in Concentration at Intervals of Two Days.*

When the concentration was increased 5 per cent. every two days instead of every week, the results were practically the same. In these experiments also the snails from Davies Lake (*D* and *E*) showed a greater resistance to sea water than the other snails.

The results of these experiments are shown briefly in Table III.

[1] For the sake of comparison the following table is given showing the average specific gravity (at 17.5° C.) and average salinity (parts per thousand) of the various concentrations of sea water used throughout these experiments. The data were calculated from Knudsen's Hydrographical Tables.

Per Cent. Normal Sea Water.	Sp. Gr. (17.5° C.).	Salinity.	Per Cent. Normal Sea Water.	Sp. Gr. (17.5° C.).	Salinity.
5%	1.0007	1	30%	1.0085	12
10%	1.0020	2 to 3	35%	1.0105	14.5
15%	1.0040	4 to 5	50%	1.0130	17
20%	1.0050	6	100%	1.0230	30
25%	1.0070	8			

TABLE II.

SHOWING EXPERIMENTS IN WATER OF HIGHER CONCENTRATION THAN 30 PER CENT.

The number of snails alive at the end of the week in 30% is given, then the number of weeks that the snails were left in water of approximately 25 or 30%; the further experiments are then summarized in a few words.

	Number Alive in 30% (from Table 1).	Number of Weeks in 25–30%.	Further Experiments.
A control.......	11		11–11
Experiment 1...	5	1	Placed in 40%; death at end of one week.
B control.......	40		40–38
Experiment 1...	5	4	Placed in 35%; one dead at end of first week; others died during second week.
Experiment 2...	5	1	Increased to 35% in which three died; increased to 50%; all died.
C control.......	83		83–83
Experiment 1...	5	2	Concentration fluctuated considerably between 1.007 and 1.012; died in four weeks.
Experiment 2...	10	2	Active in 30%; alive but inactive in 35%; died in 50%.
D control.......	50		50–50
Experiments....	9	3	Active in 30% and 35%; placed in 50% alive but inactive at end of 24 hours; revived when placed in 5% sea water.

TABLE III.

SHOWING RESULTS OF EXPERIMENTS WITH INCREASE OF 5 PER CENT. IN SALINITY AT INTERVALS OF TWO DAYS.

The number of snails alive at the beginning and end of each interval is shown

	5%	10%	15%	20%	25%	30%	35%	40%	50%
B Control...	8–8	8–8	8–8	8–8	8–8	8–8	8–8	8–8	8–8
Experiment..	5–5	5–5	5–5	5–5	5–5	5–5 fairly active	5–5 inactive	5–2 inactive	2–0 dead
C Control...	8–8	8–8	8–8	8–8	8–8	8–8	8–8	8–8	8–8
Experiment..	5–5	5–5	5–5	5–5	5–5	5–5 fairly active	5–5 inactive	5–5	5–0 dead
D Control...	21–21	21–21	21–21	21–21	21–21	21–21	18–18	18–18	18–18
Experiment..	10–10	10–10	10–10	10–10	10–9	9–9 active	9–9	9–9 fairly active	9–8 slightly active. Revived in fresh water.
E Control...	16–16	16–16	16–16	16–16	16–16	16–16	16–16	16–16	16–16
Experiment..	5–5	5–5	5–5	5–5	5–5	5–5	5–5	5–5 active	5–5 fairly active. Revived in fresh water.

Part 3, *5 Per Cent. Increase in Concentration at Intervals of One Day.*

There was no difference in the behavior of the snails when the increase was made at intervals of one day instead of two days. Those snails (*H*) from Davies Lake showed slightly greater resistance to sea water than those (*G*) from the pond in Philadelphia.

Part 4, *Sudden Change to Brackish Water.*

Physa was able to live after sudden transfers to 5 per cent., 10 per cent., 15 per cent., 20 per cent. and 25 per cent. sea water, but the Davies Lake snails (*D*, *E* and *H*) were the only ones to show signs of activity in 20 per cent. and 25 per cent. sea water.

DISCUSSION.

Whether the concentration was increased 5 per cent. at intervals of one or two days or even a week did not seem to have any significant bearing on the ability of the snails to become acclimated to the sea water. Various workers have achieved complete acclimatization of freshwater organisms to sea water over a long period of time. Beaudant (1816) successfully acclimatized *Physa fontinalis* to sea water by very gradually increasing the salinity of the water over a period of more than six months. A very long period of time, and a very gradual increase in the concentration of the salt are probably necessary for the complete acclimatization of most freshwater organisms.

Hydrogen Ion Concentration.

The possibility that death might have been caused by changes in pH, rather than by increase in salinity, was considered. Walton and Wright (1926) have shown that *Lymnæa truncatula* can stand a variation in H-ion concentration from pH 6.0 to pH 8.6, and *L. peregra* from pH 5.8 to pH 8.8. All the solutions used in the experiments lie well within this range as the following table will show:

	pH
Tap water	7.2–7.4
Aquarium water	7.2–7.4
Control after one week	7.2–7.3
Sea water from Vivarium	7.8–7.9
Sea water (Cape May Point, N. J.)	8.1–8.3
25 per cent. sea water (diluted with aquarium water)	7.5–7.7

The pH was determined colorimetrically by the use of the indicators Cresol Red and Brom Thymol Blue, and was verified in a few cases electrolytically. *Physa* was able to live normally when transferred to the acid Cedar Swamp water from the Mullica River near Batsto, N. J. (pH 5.9); likewise the snails could live in synthetic alkaline water (pH 8.1).

These data serve to show that probably some effect of the salts present in sea water rather than the change in H-ion concentration causes the death of the snails.

A More Hardy Stock from Davies Lake.

Numerous indications throughout the experiments showed that the snails from Davies Lake, Cape May Point, N. J. (controls *D*, *E* and *H*) have more resistance than the snails from the other localities. They were more active than the others in 25–30 per cent. sea water; they were active in water as concentrated as 1.012 (at 17.5 °C.), showing a resistance not shared by the other stocks; they were the only stock to survive 50 per cent. sea water; here they were inactive, but soon revived when placed in 5 per cent. sea water; they were the only stock to show any signs of activity after a sudden transfer from fresh water to 20 per cent. and 25 per cent. sea water.

This evidence seems to show fairly well that this stock is more resistant to sea water than those taken from the vicinity of Philadelphia. Although several tests of water from Davies Lake showed a specific gravity of 1.000 at 4° C., during storms the waters of the lake are probably mixed with the salt water of Delaware Bay only 50 yards distant. The resistance of the Davies Lake stock may have been acquired during several generations.

Bailey (1929) has recorded *P. heterostropha* together with other freshwater snails in the brackish water of Chesapeake Bay, and as mentioned above, the writer has found the same species in Barnegat Bay, N. J.

SUMMARY.

1. By gradually increasing the salinity, *Physa heterostropha* was made to live quite actively in 25 per cent. sea water.
2. *Physa* can live but is not active in concentrations between 30 per cent. and 40 per cent.

3. In water stronger than 40 per cent. all the snails died with the exception of the Davies Lake lot, which had retreated within their shells, but which revived when placed in 5 per cent. sea water.

4. Whether the 5 per cent. increase in concentration was made at intervals of one or two days, or even a week, seemed to make no difference in the ability of the snails to become acclimated to the sea water.

5. All the races survived a sudden change to 5 per cent., 10 per cent., 15 per cent., 20 per cent. and 25 per cent., but the Davies Lake stock was the only one active in 20 per cent. and 25 per cent., at the end of one week.

6. The snails from Davies Lake may be regarded as a stock which is more hardy to sea water than the other stocks used in the experiments.

BIBLIOGRAPHY.

Bailey, J.

1929 Fresh Water Mollusca in Brackish Water. Nautilus, Vol. 43, p. 34.

Beaudant, F. S.

1816 Memoire sur la possibilité de faire vivre des mollusques fluviantiles dans les eaux salines. Jour. de Physique, T. 83, pp. 268–284.

Crabb, Edward D.

1929 Growth of a Pond Snail, *Lymnæa stagnalis appressa* as Indicated by Increase in Shell Size. Biol. Bull., Vol. 56, pp. 41–63.

Richards, H. G.

1929 Freshwater Snails in Brackish Water. Nautilus, Vol. 42, pp. 129–30.

Walton, C. L., and Wright, W. R.

1926 Hydrogen Ion Concentration and Distribution of *Limnæa truncatula* and *L. peregra*, with notes on Mosquitoes. Parasitology, Vol. 18, pp. 363–367.

CHANGES IN pH OF ALBUMEN AND YOLK IN THE COURSE OF EMBRYONIC DEVELOPMENT UNDER NATURAL AND ARTIFICIAL INCUBATION.

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Considering the influence of acidity on colloidal properties of organic substances, such as animal fluids, tissues, etc., it is important to recognize the significance of pH value in various parts of the developing bird's egg. The most essential parts of the egg are albumen and yolk; they furnish almost all the food material to the growing embryo during the entire period of incubation. They must have a proper pH value. There is evidence to warrant the belief that any variation in pH of albumen and yolk from a normal value, particularly when affected by an environment, such as carbon dioxide gas, will develop a pathological condition within an egg and in extreme cases, will cause the death of the embryo. Therefore, it is important to establish the normal curves of pH value for both egg constituents in order to assist in further comparative studies of the growth and metabolism of the embryo under various environmental conditions of artificial incubation.

The literature relating to the subject shows that Aggazzotti, ('13), presumably, was the first who observed pH value of albumen and yolk in incubated eggs. Then a few observations were made by Gueylard ('25), Healy and Peter ('25), and, on egg albumen alone, by Wladimiroff ('26). Unfortunately, the data of the above investigators, being either insufficient or inconsistent, do not give a complete picture of all changes in pH value of the egg constituents during the incubation period.

The present paper concerns itself with a study of pH value of albumen and yolk in the developing egg throughout the incubation period under both natural and artificial (experimental) incubation.

Methods and Materials.

The eggs used were from White Leghorn hens. They were selected for uniformity of size, shape, and shell texture, and incubated soon after laying (in spring, 1929). In one experiment the eggs were incubated by the natural method, under sitting hens; in another experiment, by an artificial method, in a special electric laboratory incubator (Romanoff, '29). All physical factors of artificial incubation were predetermined and kept constant. The temperature was 38.0 ± 0.2° C.; the relative humidity 62.5 ± 1.0 per cent; the inside ventilation was 0.5 cu. ft., and fresh air from outside was added to give an adequate supply of oxygen. The carbon dioxide content was increasing from 0.15 to 0.65 per cent. The eggs were turned three times a day.

In the experiments, at intervals of twenty-four hours, usually four eggs with normally-developed embryos were removed for analysis. The pH values of egg albumen and yolk were determined electrometrically, using hydrogen electrode. The observation of yolk was carried on up to the hatching time, and that of albumen up to the sixteenth day of incubation, as long as it was available. After that time the egg albumen normally enters the yolk sac and loses its physical appearance.

Results and Discussion.

In a study of fresh eggs (just laid, warm, and without an air cell) it was found that the pH value of albumen and yolk was on an average 7.827 ± 0.046 and 5.973 ± 0.015. A slight difference was noticed in pH value of the outer and middle layers of egg albumen, examined separately. This is illustrated by the data of a few analyses (Table I.).

Table I.

Hydrogen-ion Concentration in Fresh Eggs.

Egg Number.	Layers of Egg Albumen.		Egg Yolk.
	Outer.	Middle.	
	pH	pH	pH
1	7.929	7.890	5.960
2	7.995	7.951	5.977
3	8.001	7.946	5.968
Average	7.975	7.929	5.968

The middle layer, being close to the yolk (Fig. 1), was invariably less alkaline than the outer one. In incubation as soon as an egg is heated the different layers of albumen disappear, and the separate analyses can not be performed. No attempt was made to determine pH value of the inner layer, because of its extremely small size.

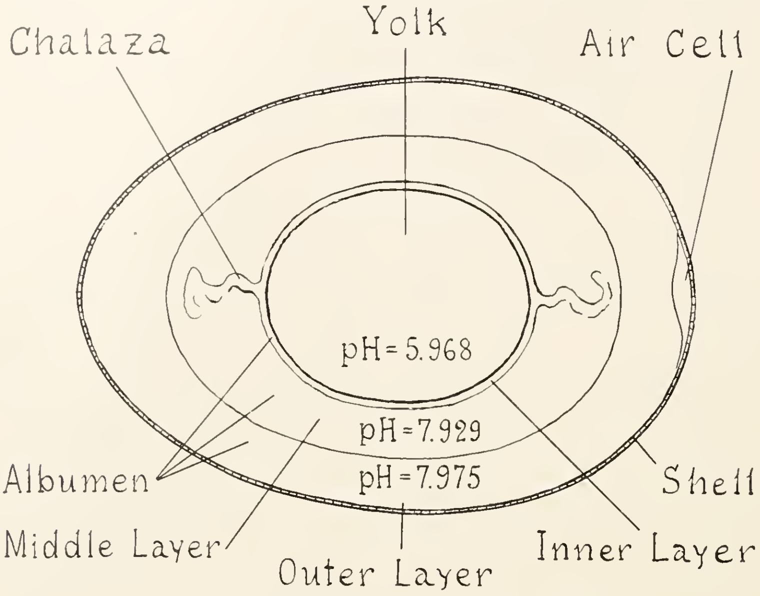

FIG. 1. Hydrogen-ion concentration of a fresh egg.

The data of our experiments on pH value of egg albumen under natural and artificial incubations, together with the data of previous workers, are shown in Table II.

It is evident, particularly from our data (Fig. 2), that egg albumen rapidly becomes alkaline, reaching the highest point at about two days of incubation. Then it turns towards neutrality, reaching the original pH value at about six days of incubation. A slight difference in pH value at two days, under natural and artificial methods, may be explained primarily by the influence of environmental conditions of incubation. Presumably, the carbon dioxide concentration was lower in the laboratory incubator than under the sitting hen. This difference can not exceed 0.1 per cent. of carbon dioxide concentration.[1]

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[1] A paper on "Effect of Carbon Dioxide on pH of Albumen in the Developing Egg," has been prepared for publication. (Will appear in *Jour. Exp. Zool.*)

The data of our experiments on pH value of egg yolk under both methods of incubation, with the data of other workers, are shown in Table III.

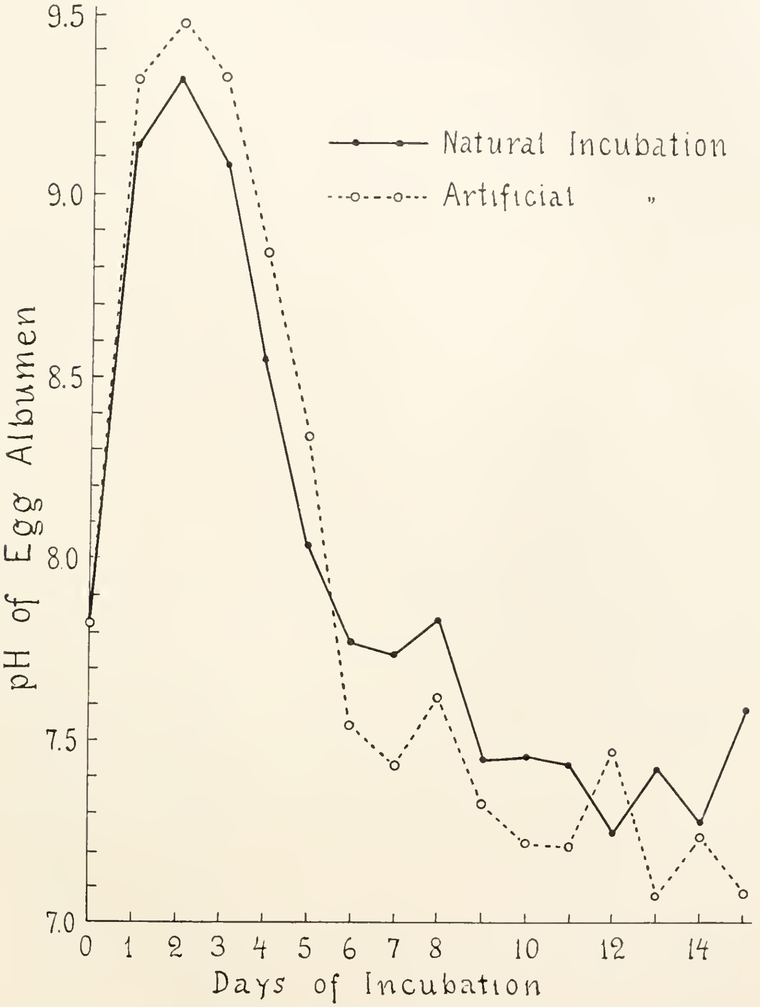

FIG. 2. Changes in pH of egg albumen under natural and artificial (experimental) incubation.

The pH value found by Aggazzotti ('13) seems to be consistently lower than ours. His data, being incomplete, do not demonstrate the pronounced drop at 16 days of incubation. It is seen

TABLE II.

CHANGES IN pH OF EGG ALBUMEN DURING INCUBATION.

Incub. Age.	Aggazzotti (1913).	Gueylard & Portier (1925).	Healy & Peter (1925).	Wladimiroff (1926).	Authors.	
					Art. Incub.	Nat. Incub.
(days)	pH	pH	pH	pH	pH	pH
0	8.66	7.97	8.24		7.827	7.827
1	8.34				9.317	9.157
2	8.38	8.60		9.36	9.472	9.311
3	7.91		9.4	9.46	9.318	9.080
4	7.40			9.19	8.835	8.546
5	7.82			8.72	8.343	8.038
6	7.41		8.2	8.66	7.536	7.785
7	7.71			8.57	7.414	7.748
8	7.24			7.88	7.614	7.840
9	7.34			8.29	7.328	7.450
10		7.25		7.82	7.255	7.466
11	6.61			7.88	7.231	7.435
12				7.55	7.473	7.257
13				7.47	7.079	7.419
14	6.14				7.247	7.283
15				7.57	7.086	7.593
16				7.61		

TABLE III.

CHANGES IN pH OF EGG YOLK DURING INCUBATION.

Incub. Age.	Aggazzotti (1913).	Gueylard & Portier (1925).	Healy & Peter (1925).	Authors.	
				Art. Incub.	Nat. Incub.
(days)	pH	pH	pH	pH	pH
0	4.06	5.48	6.36	5.973	5.973
1	4.83			6.087	6.034
2	4.04	5.73		6.151	6.082
3	4.37		6.8	6.215	6.234
4	5.55			6.286	6.117
5	5.73			6.445	6.541
6	6.12	7.54	6.8	6.631	6.589
7	5.99			6.667	6.872
8	5.96			6.756	6.946
9	5.85			6.750	7.007
10		6.83		6.855	7.327
11	6.68			6.926	7.430
12				7.050	7.450
13	6.82	7.42		7.043	7.772
14	6.98	7.08		7.042	7.920
15				6.899	8.099
16				6.105	7.401
17	6.83	4.12		7.693	7.820
18	6.26	5.64		7.701	7.637
19	6.64			7.714	7.608

from the curves of pH value under natural and artificial incubation (Fig. 3) that egg yolk, from its original acid state, gradually goes to a neutral, and then to a slightly alkaline state. The above-mentioned sudden drop in pH value under both natural and artificial incubation is possibly related to the natural depression of the growth in the life span of the embryo (Romanoff, '29*a*). But the difference in pH value under natural and artificial meth-

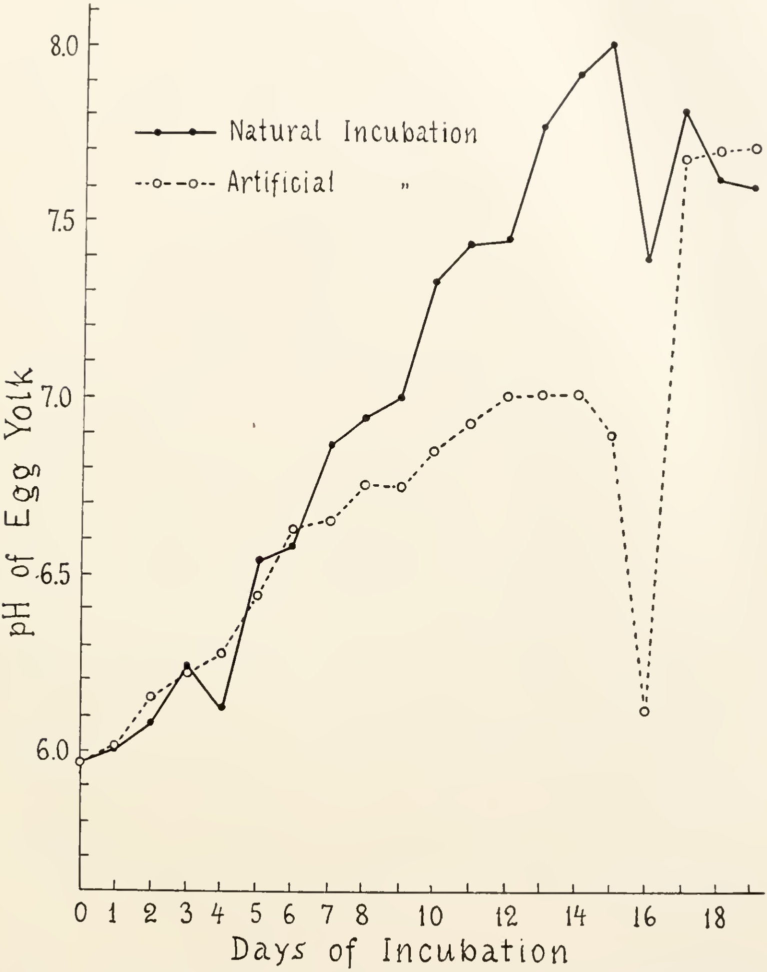

FIG. 3. Changes in pH of egg yolk under natural and artificial (experimental) incubation.

ods during the period from 7 to 16 days of incubation may be explained, as suggested above by the difference in carbon dioxide concentration.

SUMMARY.

1. In the course of embryonic development the pH values of albumen and yolk go through definite changes, presumably affected by natural metabolic processes occuring within an egg.

2. It was found that the changes in pH either of albumen or of yolk were similar under natural and artificial (laboratory) methods of incubation.

3. The pH of egg albumen rapidly changed towards alkalinity and back during the first week of incubation, reaching the highest point of alkalinity at about 48 hours. For the rest of the incubation period it gradually moved towards neutrality.

4. The pH of egg yolk gradually changed throughout the incubation period from acid to alkaline, with a sudden temporary drop at the sixteenth day.

REFERENCES.

Aggazzotti, A.

'13 Influenza dell'aria rarefatta sull'ontogenesi. II. La reazione dei liquidi dell'ovo durante lo sviluppo. Arch. f. Entw.-Mech. Organism., 37:1–28.

Gueylard, F., et de M. P. Portier.

'25 Réaction ionique des différents constituants de l'oeuf de la Poule. Ses modifications au cours de l'incubation. Compt. Rend. Acad. d. Sci., 180: 1962–1963.

Healy, D. J., and A. M. Peter.

'25 The Hydrogen-ion Concentrations and Basicity of Egg Yolk and Egg White. Am. J. Physiol., 74:363–368.

Wladimiroff, G. E.

'26 Beiträge zur Embryochemie und Embryophysiologie. I. Einige physikalisch-chemische Veränderungen des Eiereiweisses sich entwickelnder Hühnereier. Biochem. Zeitschr., 177:280–297.

Romanoff, A L.

'29 Laboratory Incubator for the Biological Study of Chick Embryo. Science, 69:197–198.

Romanoff, A. L.

'29*a* Cycles in the Prenatal Growth of the Domestic Fowl. Science, 70: 484.

BREEDING HABITS OF *NEREIS DUMERILII* AT NAPLES.[1]

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In his monograph on the natural history of *Nereis dumerilii*, Hempelmann has given us a very complete account of the life cycle of this annelid. My own work on the life history of *Platynereis* was stimulated by his classic study. During some years of experience in rearing sexually mature *Platynereis* from inseminated eggs normally laid, I was never able, under laboratory conditions, to establish for this form a life history comparable to that of *N. dumerilii.* I drew my conclusions with some reservation, stating that it might still be possible in nature that *Platynereis* goes through such a life cycle. When in addition I later made some experiments which indicated the possibility of sex reversal in sexually immature *Platynereis,* I became more sceptical of the earlier work: it might be that what appeared as sex-reversal was merely the exaggeration of the female condition, in normally hermaphroditic individuals, to the extinction of the male sex. If there were normally hermaphroditic *Platynereis* which I had previously overlooked, it was possible that I had also failed to observe other stages in the life cycle comparable to those described by Hempelmann. Finally, when Prof. J. Percy Moore suggested to me that *Platynereis megalops* and *Nereis dumerilii* might be identical, I was indeed ready to discard all of my results on *P. megalops.*

One approach to the problem seemed evident: namely, to study the breeding habits and development of *N. dumerilii* at Naples where Hempelmann had conducted his researches, although I had implicit confidence in the validity of his findings. I may say at once that so far as my studies went, they completely confirm Hempelmann's. It would have been nothing short of amazing if, for example, this painstaking worker had overlooked such an obvious and striking phenomenon as the breeding behavior exhibited by *Platynereis megalops*. After almost ten years of waiting I was able in 1929 to spend several months at the Naples Zoölogical Station with the primary object of making observations on *N. dumerilii*. Their results are here reported.

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It is a very great pleasure to take this opportunity to thank Prof. R. Dohrn, Director of the Naples Zoölogical Station, for his many kindnesses which made my stay most profitable. I also wish to acknowledge my great indebtedness to Prof. Dr. Josef Spek for placing at my disposal valuable information, the result of his own experience, concerning the care of *N. dumerilii* in the nereid phase.

The Observations.

The observations on breeding habits of *Nereis dumerilii* at the Naples Zoölogical Station during the period January to June, 1929, fall into two categories: first, observations on animals reared to sexual maturity in the laboratory; and second, those made on sexually mature animals during the swarming period.

Sexually immature specimens of *N. dumerilii* brought to the laboratory were kept in dishes of sea-water with diatoms and algæ in which the worms formed tubes. They were examined daily and isolated when found, by the appearance of the parapodia, to be nearly sexually mature. At maturity the animals were observed in pairs, the sperm and egg shedding noted.

Repeated observations of this kind made during February and March revealed that the sperm-shedding and egg-shedding processes are almost identical with those described by Lillie and Just for *Nereis limbata* at Woods Hole, Mass. Briefly, the presence of a mature female or its eggs in sea-water induces sperm shedding by the male when placed in the dish containing the female or its eggs. In three cases males shed sperm when placed in a dish with a female that externally appeared sexually mature but whose eggs neither in structure nor in fertilization capacity were physiologically "ripe"—that is, on addition of a drop of active sperm from suspensions, known by trial inseminations on eggs from other females to be in optimum condition since fertilization resulted—no egg developed. This was never found to be the case with *Nereis*.

Also, the presence of sperm in sea-water induces oviposition. Whenever ripe females were placed in a suspension of active spermatozoa, oviposition invariably followed. When this did not take place the eggs were in poor condition: they failed to show any signs of development following insemination with active sperm.

The sperm and egg shedding process of *N. dumerilii* thus closely resemble if they are not identical with those of *N. limbata*. It is interesting also to note that the eggs of the two forms show a close resemblance: in form, distribution and number of oil drops, and in pigmentation. I did not measure the eggs, but Professor Spek informed me that they are 100 μ in diameter. Eggs of *N. limbata* are about 87.5 to 100 μ. On the other hand, these sexually mature worms are morphologically different from *N. limbata*. Indeed they more closely resemble *Platynereis megalops*.

I have never seen a swarming of *N. limbata* to equal that of *N. dumerilii* at Naples after the full moon of May twenty-third. Within the circle of the light, the sea seemed alive with worms. One evening at Capri and also once at Ischia I also observed the swarming of *N. dumerilli*. During the swarming period the animals exhibit precisely the same breeding behavior described for *N. limbata*. Around the slowly swimming females, the active males move with ever increasing rapidity and more closely set spirals. Frequently, several males would thus swim over and under a female. They then discharge sperm. Into the cloud of sperm the female discharges her eggs and sinks to the bottom of the sea. In the laboratory, males and females—separated at the time of capture—when placed together, went through the same performance. *There was never any indication of the copulation so characteristic of Platynereis megalops.*

This note on breeding habits of *Nereis dumerilii* at Naples is sufficient to show that this annelid behaves almost identically as *N. limbata*. If *N. dumerilii* and *Platynereis megalops* are the same, either Hempelmann in his careful observations, made during a prolonged stay at Naples, missed that form in the life cycle of *N. dumerilii* which lays eggs only after copulation, as does *Platynereis megalops;* or, differences in the American environment are responsible for the peculiar breeding habits of *Platynereis megalops*. I am loathe to accept the first alternative; the second

I consider unlikely. It might be argued that my own work is far too incomplete to warrant any assumption that I have definitely settled this question. With this I would heartily agree. However, it is permissible, I think, to conclude that as far as they go, my observations indicate that *Nereis dumerilii* and *Platynereis megalops* are not the same species.

LITERATURE CITED.

Hempelmann, F.

'11 Zur Naturgeschichte von *Nereis dumerilii* Aud. et Edw. Zoologica, Bd. 25, Lief 1 (Heft 62).

Just, E. E.

'14 Breeding Habits of *Platynereis megalops* at Woods Hole, Mass. Biol. Bull., 27.

Just, E. E.

'22 On Rearing Sexually Mature *Platynereis megalops* from Eggs. The American Naturalist, 56.

Lillie, F. R., and Just, E. E.

'13 Breeding Habits of the Heteronereis Form of *Nereis limbata* at Woods Hole, Mass. Biol. Bull., 24.

THE PRODUCTION OF FILAMENTS BY ECHINODERM OVA AS A RESPONSE TO INSEMINATION, WITH SPECIAL REFERENCE TO THE PHENOMENON AS EXHIBITED BY OVA OF THE GENUS *ASTERIAS*.[1]

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In 1923 Chambers recorded results which constituted "an attempt to explain the peculiar behavior of the starfish spermatozoön which enables it to migrate through the jelly of the egg." On the basis of my observations extending over a period of years on living and sectioned eggs of the genus *Asterias*, I was forced to disagree somewhat with Chambers' results. The main point of this disagreement concerned itself with Chambers' time-table of the events in the process of sperm entry which follow insemination of the egg. His observations I may briefly summarize as follows:

Twenty-seven minutes after insemination the spermatozoön at the outer boundary of the egg jelly is in contact with a fine filament which grows out from a conical elevation on the surface of the egg through the intervening jelly. The spermatozoön approaches nearer the egg surface and reaches the cone; one gains the impression that the cone exerts a pull on the spermatozoön (two minutes after insemination.) With the arrival of the spermatozoön at the summit of the cone, there ensues a pause of about thirty seconds. The sperm-head narrows at its tip, lengthens, and rapidly slips through the vitelline ("fertilization") membrane which, one minute after insemination, had separated from the egg surface (two minutes and thirty-three seconds after insemination). Within the substance of the cone the sperm-head rounds up (two minutes and thirty-four seconds after insemination).

On the other hand, I have repeatedly observed in both the living and sectioned eggs (killed during the period of three minutes

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after insemination at five second intervals) that: (1), spermatozoa have reached the egg surface through the jelly *five seconds* after insemination; (2), spermatozoa are well attached to the vitelline membrane *fifteen seconds* after insemination; (3), the vitelline membrane begins to separate from the egg surface *forty-five seconds* after insemination at which time the entrance cone is well defined within the perivitelline space; and (4), the spermatozoön enters the cone about *two minutes* after insemination. With respect to sequence of events in the process of sperm entry, my data are thus in serious conflict with those of Chambers. The question was therefore raised whether the manipulations of the ova inherent in his methods of examination might not have produced abnormal behavior. (See Lillie and Just, 1924, pp. 458–559.)

In my judgment, however, there is an equally serious question, quite apart from that of the micro-dissection method, concerning this work of Chambers on the mechanism of the entrance of the spermatozoön into the starfish egg. Though the main interest throughout my studies of fertilization in the genus *Asterias* has been the *normal* process of sperm entry in ova in optimum fertilizable condition, I have from time to time noted the production of filaments in ova of this and other echinoderms as a response to insemination. Such productions, I believe, are the response of abnormal ova. The present communication aims to set forth the evidence on which I base this belief.

OBSERVATIONS.

The formation of filaments (or papillæ) as a response to insemination have been observed in ova of the following echinoderms: *Thyone briareus*, *Strongylocentrotus dröbachiensis*, *Arbacia punctulata*, *Asterias forbesii*, and *A. vulgaris*. The observations were made during several spring and summer months at the Marine Biological Laboratory, Woods Hole, Mass. More recently, during the winter months of 1929, while enjoying the privilege of working at the Zoölogical Station of Naples, I also made observations on the ova of *Asterias glacialis*.

We may begin the description of the production of filaments by ova as a response to insemination with an account of the phenomenon in the ovocytes of *Thyone briareus*.

Eggs of *Thyone* are readily inseminated if they have been naturally shed by the animals. The animals, easily obtainable in abundance, spawn actively throughout the breeding season, provided they are kept in large quantities of sea-water. Pearse ('09) has recorded an observation on the spawning of *Thyone*. These normally shed eggs, as observations made repeatedly during the month of June of several years revealed, never form protoplasmic filaments as a response to insemination. During April and May, 1927, however, I was always able to observe the extrusion of filaments from the cortex of *ovocytes* on insemination. In most cases the surface of the ovocytes literally bristled with the delicate filaments extending beyond the striated egg-jelly. But these ovocytes never developed.

The formation of protoplasmic filaments by holothurian ovocytes in the presence of spermatozoa has been previously reported by Hobson who studied the phenomenon in *Holothuria nigra*.

Strongylocentrotus dröbachiensis. During the spring months of 1927 and 1928 I observed the formation of filaments by the mature eggs of this echinid on the addition of spermatozoa to the water containing them. These were the most beautiful and numerous filaments that I have ever seen. However, in 1927, of the eggs from thirty-three females, less than 0.7 per cent. cleaved; and no egg went beyond cleavage. The experience of 1928 was the same. The explanation is simple: the animals came to me from cold sea-water, forty-eight hours after collection, closely packed in wet sea-weed. When the inseminations were made in sea-water of a higher temperature, the gametes were either moribund or dead. At neither Woods Hole nor Naples have I ever observed filaments on mature echinid ova capable of fertilization. But stale eggs no longer capable of fertilization do produce them.

The indications are that this response of mature *Strongylocentrotus* eggs to insemination is due to the abnormal condition of the gametes. This position is supported by the observations of Mrs. Andrews which reveal that mature echinid eggs in an abnormal condition—produced by heat, pressure, or confinement—exhibit a cortical behavior markedly different from the normal. It is certain that Mrs. Andrews observed the production of filaments by *Echinus* eggs.

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The literature affords no more beautiful observations on living eggs than those recorded by Mrs. Andrews. Her papers are marvels for their painstaking and accurate descriptions of the delicate and ever-changing cortical phenomena which echinoderm eggs exhibit throughout their development. Her meticulous account of the spinning processes which establish the hyaline plasma layer of echinid eggs after insemination, for example, has never been equalled; in comparison the descriptions by subsequent workers, who have completely ignored her work, are banal, superficial, and pitifully inadequate. It is most unfortunate that Mrs. Andrews' work has not received the recognition it so richly deserves.

Arbacia punctulata. Ovocytes of *Arbacia* when inseminated form processes which fifteen to twenty minutes after insemination are blunt papillæ much larger than the entrance cones found in normal fertilization. Wilson has described and photographed ovocytes of *Toxopneustes* showing these cones, as he named them. In addition, Seifriz has at some length described filaments on ovocytes of *Echinarachnius.* Runnström (1928) has given a review of the work on this phenomenon. Further comment is therefore unnecessary.

From the foregoing summary one is justified, I think, in reaching the conclusion, at least for the forms named, that cortical filaments as a response to insemination are produced only by eggs incapable of the fertilization-reaction: either the eggs, though in the fertilizable stage, are abnormal or they are immature. The question therefore arises concerning the production of filaments by eggs of *Asterias.* Is it possible that here also the phenomenon, as described by Chambers, is due not merely to the injury of the eggs incident to the micro-dissection methods which he employed, but also—and this is more cogent—to their abnormal condition? I believe that the evidence which I shall now present indicates that he did use abnormal eggs.

Asterias. In the first place, eggs of *Asterias forbesii* and *A. vulgaris* in the germinal vesicle stage produce cortical filaments on insemination. Such eggs never develop. Indeed, insemination, like treatment with butyric acid or heat (R. S. Lillie, 1914), inhibits maturation even. At Naples I found that eggs of *A.*

glacialis in the germinal vesicle stage react to insemination in a similar way. Hobson previously had noted the same phenomenon in *A. rubens*. Says Hobson: "Protoplasmic papillæ are found, so similar in their structure and behavior to those in *Echinarachnius* that, although these observations were made before I had seen Seifriz's paper, their repetition would simply duplicate his study." These eggs never complete their maturation or cleave, according to Hobson. He further suggests that this formation of protoplasmic papillæ "will be found to be widely spread in Echinoderms since the present writer has also observed similar formation of protoplasmic papillæ by unripe oocytes of *Holothuria nigra* in the presence of sperm."

In the second place, after the break-down of the germinal vesicle when the eggs are fertilizable or capable of responding to agents of experimental parthenogenesis, it is also possible to obtain cortical filaments as a consequence of insemination. In this case, however, the egg must be subjected to some form of injury: *e.g.*, pressure, overcrowding, CO_2, or increase in temperature. After complete maturation with the drop in fertilizability insemination likewise induces the formation of filaments.

(*a*) If eggs of *Asterias* which, after the rupture of the germinal vesicle, are capable of normal fertilization, maturation, cleavage, and embryo formation be inseminated under the pressure of a cover-slip, filaments are formed in abundance. For example, a very fine lot of eggs, taken from the ovaries of a shedding female, gave 99 per cent. cleavage, beautiful gastrulæ, and vigorous larvæ —in no wise to be distinguished from the larvæ produced from shed eggs. 0.2 cc. of these eggs inseminated, in finger bowls containing 200 cc. of sea-water, at ten minute intervals after removal from the ovaries to sea-water up to the time of the extrusion of the second polar body, showed no filaments. On the other hand samples of these same eggs inseminated under the pressure of a cover-slip did show filaments.

(*b*) Approximately 100 eggs were inseminated in ten cc. of sea-water at ten minute intervals after removal from the ovaries as in (a). Every egg cleaved normally. On the other hand, similar lots from the same ovaries each inseminated in a drop of water on a slide formed filaments and gave extremely low percentages of abnormal development.

(*c*) Eggs from one female were divided into two dense suspensions of 50 cc. each. *Lot A:* eggs were placed in 5 liters of sea-water. *Lot B:* eggs were placed in 150 cc. of sea-water. Thirty minutes later a drop of eggs from each lot was examined under the microscope: 90 per cent. of the eggs of *Lot A* were in process of maturation; 90 per cent. of the eggs of *Lot B* showed germinal vesicles intact. Eggs of *Lot A* on insemination showed no filaments, but those of *Lot B* whether with or without intact germinal vesicles did. 95 per cent. of the inseminated eggs of *Lot A* gave vigorous larvæ, while less than 1 per cent. of *Lot B* gave normal gastrulæ.

This observation is by no means an isolated one. Experience has shown that large volumes of sea-water are indispensable for the break-down of the germinal vesicle and therefore for optimum fertilization capacity as well as for experimental parthenogenesis. Eggs taken from the ovaries of a shedding female will yield a very low per cent. of maturation if crowded; the same eggs show close to 100 per cent. maturation in large dishes of sea-water. This point merits more than passing notice.

My first experience with the living egg of the starfish dates back to 1909. Repeated attempts to obtain viable larvæ from eggs inseminated after removal from the ovaries were failures. In June 1910, thanks to Dr. John W. Scott, who very generously gave me a male and a female, both of which were actively shedding, I was able to carry through my first lot of eggs to the brachiolaria stage. In 1911 I carried normally shed eggs inseminated with normally shed sperm through metamorphosis. Since then I have frequently obtained shedding animals, the eggs of which always gave a high per cent. of normal membrane separation, maturation, cleavage, and bipinnaria. These larvæ if fed metamorphosed. Indeed, during some years, notably June 1927, the ready shedding of the animals was actually a nuisance: animals procured in late afternoon often shed in the early morning hours and the eggs when removed from the tanks were found in cleavage stages.

Because of the extensive experimental work done on starfish eggs, it very early occurred to me that it might be of interest to learn whether normally shed eggs—as measured by the fertilization-reaction, by the rate, uniformity, and per cent. of cleavage and by the size and viability of the larvæ, etc.—are superior to eggs obtained directly from the ovaries, according to the method which most workers employ. A moment's consideration suffices to show the significance of such a study: from it one might establish a basis for appraisal of all experimental work done on these eggs which fails to consider the normal development of normally shed eggs. Too generally, I think, workers have made experiments on starfish eggs without knowledge of the development of shed eggs.

Now the eggs shed by starfish are at once fertilizable. These eggs give practically 100 per cent. cleavage and beautiful vigorous larvæ of uniform size. I found that eggs taken from ovaries, which must stand in sea-water until breakdown of the germinal vesicle before insemination, are often inferior as revealed by the lower per cent. of cleavage and less viable larvæ of varying sizes. This seemed serious to me not only because it might cast suspicion on much of the experimental work done on such eggs but because it would seem to nullify my main object in using eggs of the starfish: namely, to study their fertilization in every stage of the maturation processes as a basis of comparison with those eggs in which the fertilization-moment is more rigidly fixed with respect to the stages in maturation. I soon found, however, that it is possible to obtain starfish eggs from the ovaries in every way equal to those that are naturally shed. The procedure is simple:

I either take the eggs from the ovaries of shedding females or, selecting the heaviest and most healthy-looking animals, remove the eggs from the ovaries at once. In either case I use at least three liters of sea-water in a dish of about 28 cm. in diameter to which I add either *one* ovary carefully exposed and removed or only the broad end of the ovary next the disc. After five minutes the ovary is removed. One drop of dry sperm is placed in 200 cc. of sea-water in which they are intensely active.

This method has given me most excellent results. On the other hand, eggs from unselected females concentrated in a small volume of sea-water, on insemination not only show cortical filaments, but also yield a small per cent. of abnormal cleavage and larvæ of low viability.

Fol, in his classic monograph on fertilization, emphasized the importance of using fresh animals with distended ovaries. He also advised using large quantities of sea-water. In addition he cautioned, for both the sea-urchin and starfish, against contamination of the eggs by the cœlomic fluid.

(*d*) CO_2 and heat were each found capable of causing the production of filaments by eggs in the optimum fertilizable condition. If, for example, eggs, with the germinal vesicle broken down, which give 90 or a greater per cent. of membrane separation be inseminated after treatment for one minute with sea-water, charged with CO_2, they form filaments. Or, if such eggs taken from sea-water at 15°–16° C. be inseminated in sea-water at 26° C., they form beautiful filaments.

The foregoing brief account of the observations on eggs of the genus *Asterias* indicate (1) that in the germinal vesicle stage when incapable of fertilization they produce filaments as do immature eggs of *Thyone*, *Arbacia*, *Toxopneustes* (Wilson), *Echinarachnius* (Seifriz), *Holothuria nigra* (Hobson), *Asterias rubens* (Hobson); and (2) that in the fertilizable stage, after break-down of the germinal vesicle, if inseminated under adverse conditions, they produce filaments.

In the next place, after complete maturation the fertilizability of *Asterias* eggs falls off. During May and June of 1921, at Woods Hole, a careful study was made of this drop in the fertilizability of the eggs both normally shed and those taken from the ovaries. At the Naples Zoölogical Station this was found to be true for *A. glacialis* also. With the falling off in fertilizability, it was learned in 1926 and 1927, that the capacity of the eggs to produce filaments increases.

Observations on the production of filaments by completely maturated eggs as a response to insemination were among the first ones made. The eggs used fall into three classes: those normally shed, those from the ovaries of shedding females, and those from selected females with greatly distended ovaries. Of all three classes the eggs were in the optimum condition— as revealed on insemination, after break-down of the germinal vesicle, by the rate and quality of membrane separation, the regularity of cleavage, and the vigor of the larvæ. That is, eggs were inseminated at ten minute intervals after coming into sea-water throughout the maturation process in order to fix the optimum moment for fertilization. With the completion of maturation, the inseminations were continued for three to ten hours longer, the production of filaments being noted at each insemination. No lot of eggs showing more than 10 per cent. intact germinal vesicles was ever used; nor was any observation made on eggs, samples of which gave less than 90 per cent. membrane separation. In other words, the maturated eggs that produced filaments were always from lots which, inseminated at some stage of maturation, gave optimum developments.

In August 1923, at Woods Hole, soon after the appearance of Chambers' paper on the mechanism of the entrance of the spermatozoön into the starfish egg, I was fortunate in collecting some very fine shedding *Asterias* which I used immediately. Again it was found that after maturation the fertilization capacity of the eggs is lost; with this loss, the production of filaments is increased. On the other hand, eggs inseminated as shed, with the germinal vesicle broken down, were immediately fertilizable—without filaments unless adversely treated—and gave beautiful development. On January 30, 1929, at the Naples Zoölogical Station I procured my first shedding *A. glacialis;* in Feburary also I had shedding females. These gave the same results obtained at Woods Hole.

Briefly, the observations indicate that maturated eggs lose capacity for normal fertilization and parallel with this loss runs the production of filaments. Three hours after the extrusion of the second polar body (*A. forbesii* and *A. vulgaris*) filament formation is first most readily noted. On *A. glacialis* (observations on eggs of four shedding females only) the time is about three and one-half hours. Until their death the eggs respond to insemination with the production of filaments.

DISCUSSION.

If the observations given above be correct we may conclude that the production of filaments by *Asterias* ova, as well as of the other echinoderm ova studied, as a response to insemination is a phenomenon quite apart from the behavior in the normal fertilization-reaction. It follows, therefore, that any generalizations concerning its significance for the mechanism of sperm penetration into the animal egg must be tempered. Though the reasons for this statement seem fairly obvious, it may not be amiss to elaborate them somewhat. In addition, there is the primary need to consider specifically the case of the starfish egg. We may begin with Fol's original descriptions.

In his classic monograph on fertilization, Fol gave a detailed description of the entrance of the spermatozoön into the egg of *Asterias*. According to this account, several spermatozoa reach the outer border of the jelly hull; one gets through and moves toward the surface of the egg. The proximity of this spermatozoön causes the egg cortex to form a hyaline elevation, the *attraction cone*. The form and especially the length of the attraction cone vary, depending upon the rapidity with which the spermatozoön approaches the vitellus; if the approach be slow, the cone is prolonged as a filament whose length is half the diameter of the jelly hull; if the approach be rapid, the spermatozoön reaches the cone before it can elongate. The form of the cone thus depends upon the activity of that spermatozoön which first pierces the jelly hull (Fol, page 91).

As noted above, I have made observations on the penetration of the spermatozoön into the normal starfish egg in the optimum stage for fertilization. These observations have been supplemented by study of several series of sectioned eggs fixed at 5 or 10 second intervals beginning at 5 and ending at 120 to 180 seconds after insemination. In each series the eggs were from a single female and inseminations at 10 or 15 minute intervals from the time they were brought into sea-water until complete maturation; thereafter inseminations were made at 30 minute intervals for two or three hours. Thus, one series alone makes up over 200 stages of sectioned material. The rapidity of normal sperm attachment, due to the intense activity of the spermatozoa in optimum conditions, demands a study of fixed material despite the importance of observations on living eggs and the present-day vogue of disparaging the study of fixed cells.

On the basis of these observations I am forced to disagree with Fol's account of the formation of an attraction cone stimulated by proximity of the spermatozoön to the egg surface. As I see it, the phenomenon is as follows:

The intensely active spermatozoa rush toward the jelly hull; of these, one rapidly moving through it reaches the egg within 5 seconds after insemination. *The cone forms after the attachment of the spermatozoön to the vitelline membrane.* As the cone grows the spermatozoön—seen in both living and fixed preparations—is pushed off from the egg, a delicate strand connecting the tip with the apex of the cone. This strand never attains the length given by Fol, as half the diameter of the jelly hull, and of course could never therefore be equal to the greater length figured by Chambers. *Moreover, this strand is a prolongation of the spermatozoön, the tip of which is fixed within the cone.*

The point, which elsewhere (Lillie and Just) has been emphasized, is still considered the important one: namely, that before formation of the cone, the spermatozoön has been attached to the vitellus for about 45 seconds. The cone, whether blunt or elongated, is not, therefore, an attraction cone. Fol perhaps used too dense sperm suspensions which were above the level for optimum activity—since starfish spermatozoa are most highly active only in thin suspensions—though possessing fertilizing power and exhibiting increased activity when mixed with the eggs. But if he did this, it was not his greatest error. The main objection to his work lies in another direction.

Now Fol knew that eggs of *Asterias* are capable of insemination during first maturation. Such eggs subsequently show both polar bodies adhering to the vitellus separated from the membrane by a wide perivitelline space. They develop normally. Unfortunately Fol gave scant notice to this point, dismissing it with a few sentences. Instead, he focused his attention on the fertilization process in fully matured eggs. This meant, of course, that the eggs had been lying in sea-water for some time—about four hours at a temperature of 12° to 15° C. In my experience at least, after complete maturation, eggs of *Asterias glacialis* at Naples lose their capacity for normal fertilization; the character of the cleavages and the viability of the larvæ as well as the rate and quality of the fertilization-reaction reveal this. They therefore resemble eggs of the American species studied. Pressure, elevation of temperature, overcrowding—any one of these factors induces the formation of filaments. But we may here waive them all: *Fol used stale eggs, i.e.*, eggs that had passed the optimum condition for fertilization and this fact alone accounts for his results on the penetration of the spermatozoön.

That Fol's observations are correct I do not for a moment dispute, nor do I deny Chambers' similar findings—I have too often seen the cortical phenomena which they described. What I do contend is that their account of the cone (or filament) formation belongs in the same category with those on immature unfertilizable eggs, fertilizable eggs rendered unfertilizable by brutal treatment, and stale eggs having passed the fertilizable period. If this contention be sustained, neither Fol's nor Chambers' work has any direct value for the normal fertilization-reaction *per se*. Nevertheless their work on abnormal eggs like that by others cited in this paper is of interest: it contributes additional testimony concerning the reactivity of the egg cortex.

There is available a sufficiently large body of evidence which indicates that the cortex (or ectoplasm) plays an important rôle in cellular activity. Whether the cells be amœbæ, egg cells, or tissue cells—*e.g.*, striated muscle or nerve of vertebrates—their surfaces are the seat of changes important for vital processes. These are not merely physico-chemical changes easily reducible to mathematical formulation which apply to non-living films of molecular dimensions. Rather, the cortex—I speak now especially of ova—is an easily visible structural entity of never-ceasing, well-defined changes. The reactivity of the cell as a whole—its individual and peculiar response to stimulation with attendant measurable physical and chemical changes—is largely, if indeed not wholly, a cortical (ectoplasmic) phenomenon. Cortical changes in ova are, therefore, no mere epiphenomena: they constitute the *sine qua non* of cellular life. In responding to and propagating the effects of the initial event in the fertilization-reaction, the attachment of the spermatozoön, the egg exhibits cortical changes wihch eventually modify the whole protoplasm and direct the course of ontogeny. The spinning activity of the cortex, to borrow Mrs. Andrews' happy phrase, is thus of great significance. Even that shown by eggs incapable of the normal fertilization-reaction, with which we are here concerned, has some value.

In the first place, these filaments formed by abnormal ova as a response to insemination are exaggerated entrance cones. Their formation is additional proof, if such be needed nowadays, that the penetration of the spermatozoön may be a phenomenon quite apart from fertilization *per se*. In the case of the immature egg, the production of filaments suggests that the mechanism for sperm entry is already laid down; in the case of the brutally treated normal, or of the stale, egg, that this mechanism persists. In all these three kinds of eggs the cortical response to insemination is diffuse and slow; it thus differs markedly from the sharply localized and rapid response of the normal egg in optimum fertilizable condition. Nor yet is the essential characteristic of the fertilization-reaction the formation of the sperm aster.

Years ago one thought of the essential phenomenon in fertilization as the introduction into the egg by the spermatozoön of centrosomes with attendant sperm aster formation. But Boveri showed—what subsequently Brachet has ponderously described, without, however, mentioning Boveri's work—that a spermatozoön may enter an echinid egg during maturation and form an aster. The egg, however, is not fertilized. It does not develop. Evidence indicates that sperm aster formation is due to the diffusion into the cytoplasm of material escaping from the broken-down germinal vesicle. Such asters form, for example, in eggs which sperm normally penetrate in the ovocyte stage when later this material is present in the endoplasm.

On the one hand, then, the egg lays down in its cortex the mechanism for sperm entry; on the other, it possesses aster-forming substances diffusing from the germinal vesicle into the cytoplasm. Neither is alone, nor are both together, sufficient for fertilization. There must be present in addition the fertilizin of Lillie at or in the cortex. Normal fertilization means the cortical reaction between spermatozoön and fertilizin which sets up the rapid explosive wave which, beginning at the point of sperm entry, sweeps over the egg surface.

In a more general way we must now consider the production of filaments by ova as a response to insemination.

Animal ova may be variously classified according to one or another of several criteria obtained through a study of their fertilization processes. Such classifications are useful, as I have elsewhere pointed out (Just, '22), for an analysis of the fertilization-reaction; for with them we distinguish between the incidental phenomena and the sequelæ of the fertilization-reaction as well as the indicia of cell division, on the one hand; and the significant event in the fertilization-reaction *per se* on the other. Thus we may come to recognise the common factor in the fertilization process of all animal ova. Now one such classification of ova, with respect to their fertilization, we may make with sperm entry as the criterion: some ova possess micropyles, others do not; some form entrance cones, others do not. Also it has been held that some form attraction cones. The entrance cone, one understands of course, forms *after* the attachment of the spermatozoön to the egg surface. In my judgment, the evidence in favor of the formation of an attraction cone, *i.e.*, an elevation of the egg cortex *before* sperm attachment is extremely doubtful. If the observations here reported be correct, then the most generally accepted case, that of *Asterias* egg, is discredited. This too, we must conclude, in normal fertilization is an entrance cone.

This cone in *Asterias* egg closely resembles that in *Nereis* egg except that the duration of time between sperm attachment and its formation and between the latter and sperm engulfment are both much longer in *Nereis* egg. Moreover, the strand between the sperm head and the cone in both cases has the same origin, namely, from the spermatozoön itself. In echinid eggs, on the other hand, cone formation and sperm penetration are more rapid than in the starfish egg. Essentially, however, the process is the same in the three types. The retraction of the cone in the eggs of *Nereis* and of *Asterias* is very similar; whereas in echinid eggs the cone persists after the spermatozoön has traversed the cortex.

The fact that many ova do not possess cones renders it unlikely that their formation is an essential phenomenon for fertilization. Even if the mechanism of sperm entrance as described by Fol and by Chambers were correct, it would thus have only limited application for fertilization processes generally. And were it possessed of general application, it would, in view of the observations on the production of filaments by eggs of feeble or no fertilization capacity recorded above, give us little aid in the analysis of the fertilization-reaction. The rapidity, the irreversibility, and the specificity of this reaction indicate a phenomenon common to all metazoan ova of far more fundamental significance than mere sperm entry. It is through the study of this common factor that we must hope to approximate more closely the solution of the fertilization problem.

LITERATURE CITED.

Andrews, G. F.

'97 Some Spinning Activities of Protoplasm in Starfish and Echinid Eggs. Jour. of Morph., Vol. 12, pp. 307–389.

Chambers, R.

'23 The Mechanism of the Entrance of Sperm into the Starfish Egg. Jour. Gen. Physiol., 5, 821–829.

Fol, H.

'79 Recherches sur la Fécondation et le Commencement de L'Henogenie Chez Divers Animaux. Memoires de la Soc. de phys. et d'hist. nat. de Genève, Vol. XXVI.

Hobson, A. D.

'27 A Study of the Fertilization Membrane in the Echinoderms. Proceedings of the Royal Soc. of Edinburgh 47.

Just, E. E.

'22 Initiation of Development in the Egg of *Arbacia*. II. Fertilization of Eggs in Various Stages of Artificially Induced Mitosis. Biol. Bull., 43, pp. 401–410.

Lillie, F. R.

'14 Studies of Fertilization. VI. The Mechanism of Fertilization in *Arbacia*. Journ. Exper. Zoöl., 16, 523–590.

Lillie, F. R. and Just, E. E.

'24 Fertilization. In General Cytology, University of Chicago Press.

Lillie, R. S.

'15 On the Conditions of Activation of Unfertilized Starfish Eggs under the Influence of High Temperatures and Fatty Acid Solutions. Biol. Bull., 28, 260–303.

Pearse, A. S.

'09 Autotomy in Holothurians. Biol. Bull., 18, 42–49.

Runnström, J.

'28 Plasmabau und Determination bei dem Ei von *Paracentrotus lividus*. Roux's Archiv 113, 556–581.

Seifriz, W.

'26 Protoplasmic Papillæ of *Echinarachnius* Oöcytes. Protoplasma 1.

Wilson, E. B.

'95 Archoplasma, Centrosome and Chromatin in the Sea Urchin Egg. Jour. Morph., 11, 443–478.

THE FERTILIZATION-REACTION IN EGGS OF *PARACENTROTUS* AND *ECHINUS*. [1]

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During three months of my stay at the Naples Zoölogical Station I had the opportunity, beginning January 25, 1929, of studying fertilization in the eggs of two echinids—*Paracentrotus lividus* and *Echinus microtuberulatus*. The work carried out on the eggs of these forms was largely a repetition of work previously done on the eggs of *Echinarachnius* and of *Arbacia*. Since certain of the results obtained on the eggs of these two American species have been used in support of Lillie's work, and since it has been suggested that some of the results are peculiar and not of general significance for the analysis of fertilization, it was felt that any data on European forms should be noted. The work was done most intensively during the month of February, following preliminary observations made during the last week in January. During March and April also observations were made. Observations were likewise made on the eggs of *Arbacia;* since the findings here were so closely similar to those obtained on the American species, no reference is made to them.

It gives me great pleasure to acknowledge my indebtedness to Prof. R. Dohrn., Director of the Naples Zoölogical Station, and to other members of the staff for unfailing courtesies which they extended me throughout my stay.

THE OBSERVATIONS.

The observations on the eggs of *Paracentrotus* and *Echinus* fall into three groups: first, observations on the rate of membrane separation; second, observations on the sperm agglutination capacity of the egg-sea-water; and third, observations on the inhibitory action of the perivisceral fluid.

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[1] From the Naples Zöological Station, and the Department of Zoölogy, Howard University, Washington, D. C.

It will be convenient to record these observations on the eggs of *Paracentrotus* and *Echinus* in turn.

Paracentrotus.

Following insemination eggs of *Paracentrotus* show membrane separation beginning in 30 seconds. This was learned after repeated observations on the eggs from single individuals as long as the eggs remained viable after removal to sea-water. From January 25 to January 30 inclusive, for example, over a hundred records were made. These results were subsequently confirmed. Membrane separation in this egg is easily followed. Beginning at the point of sperm entry, it separates from the surface of the egg until finally entirely off; the membrane then becomes equidistant from the egg surface at all points. The cortex beneath the membrane is clearly defined, being made up of delicate papillæ which eventually are covered with a delicate membrane. Papillæ and membrane constitute the hyaline plasma layer. The fertilization cone which forms around the entering sperm is pronounced and easily visible. This fact makes it easy to mark the entrance point of the spermatozoön. In addition, eggs rapidly fixed at the moment that membrane separation begins show spermatozoa attached to the eggs in the zone of membrane separation.

Observations on the inhibitory action of perivisceral fluid reveal that in some cases inhibition may be complete. For example, lots of eggs from the same female whose eggs, thoroughly washed in sea-water, on insemination in sea-water had given 95 or a greater per cent. of fertilization, inseminated in the presence of perivisceral fluid showed no membrane separation or other signs of development. Such eggs in perivisceral fluid, however, after thorough washing in sea-water, provided the washing did not extend over six hours, were fully capable of fertilization. After about six hours in normal sea-water, fertilization capacity drops as shown not only by the slow rate of membrane separation but also by the small per cent. of cleavage.

Eggs of *Paracentrotus* allowed to stand in normal sea-water produce a sperm agglutinating substance. The power of this substance varies, depending upon the maturity of the ovaries.

The best results were obtained with eggs that were normally shed or with those from animals which exuded eggs when cut. Where eggs were taken directly from the ovaries, whether or not contaminated with perivisceral fluid, which might or might not inhibit fertilization, the agglutination tests were never so sharp.

Compared with *Echinarachnius* and *Arbacia* the egg of *Paracentrotus* is inferior to *Arbacia* for the study of inhibition to fertilization by the perivisceral fluid and for agglutination tests. This I attribute to the fact that one ripe *Arbacia punctulata* yields more eggs. With respect to membrane separation it is far superior to the American species of *Arbacia* in which the process is discerned with difficulty. In *Paracentrotus* it is remarkably clear. The reader will recall that Fol years ago observed the separation of the membrane in eggs of *Paracentrotus* beginning at the point of sperm entry. On the other hand, while the process of membrane separation is more rapid than that in *Echinarachnius* the egg is a better object for study because it is more hardy.

Echinus.

In eggs of *Echinus* the first indication of membrane separation is a wrinkling of its surface. This takes place before actual separation begins. The longest time between insemination and the beginning of membrane separation noted was 100 seconds; the shortest time, 45 seconds. The average time for all of the experiments was between 45 and 50 seconds. In general the speed of separation may be correlated with fertilization capacity. For example, whenever membrane separation was delayed for 100 seconds about 50 per cent. of the eggs showed no membrane separation whatsoever. In one such lot of slowly reacting eggs, which in seven trials gave the time between insemination and membrane separation between 95 and 100 seconds, heavy insemination induced membrane separation only after 180 seconds. The membrane is completely off and equidistant from the egg at all points 150 seconds after insemination. In this egg vesicles comparable to those noted in *Echinarachnius* were found in the perivitelline space. The cortex under the membrane is well marked. I may note in passing that eggs in the germinal vesicle stage whenever found always responded to insemination by forming papillæ (C. F. Runnström, 1924 for *Psammechinus miliaris*).

The presence of perivisceral fluid strongly inhibits fertilization. When the animals are used soon after collection their eggs give remarkably uniform results with respect to membrane separation, cleavage, and later development. I was frequently able to keep plutei in the laboratory for two weeks after insemination. The inhibition to fertilization tested as previously in the work on other forms (Lillie, '14, Just, '22, '23) could not therefore be attributed to the quality of the gametes.

Uninseminated *Echinus* eggs in sea-water produce a sperm agglutinin. The production of the agglutinin runs parallel with fertilization capacity. Of two lots of eggs, for example, from two different females, one producing strong agglutinating substance and the other a weak, membrane separation, per cent. of cleavage, and normality of later development are superior in the former. On February 21, observations were made on the eggs from ten females. In each case high agglutinin production was correlated with optimum fertilization capacity. Throughout the course of the observations eggs of low agglutinin output gave poor response to insemination.

DISCUSSION.

Fol years ago noted the fact that following insemination the vitelline membrane begins to separate from the surface of the egg of *Strongylocentrotus* (*Paracentrotus*) at the point of sperm entry. This is likewise true for eggs of *Echinarachnius* (Just, '19) and *Arbacia* (Just, '28). The observations here reported thus confirm earlier ones. The egg of *Arbacia* is the most difficult object for the observation of this phenomenon; nevertheless study of both the living and the sectioned egg establishes the fact. On the other hand, there is no difficulty whatsoever in following the sequence of events between insemination and membrane separation if one employs any of the three other species named. Any worker possessing only mediocre powers of observation, therefore, should be able to prove to his own satisfaction that eggs, of these three forms at least, separate membranes, beginning at the point of sperm entry. This is a question not of argument or "interpretation"; rather, it is merely a matter of being able to make a simple observation.

The appearance of vesicles in the perivitelline space is worthy of note. As the membrane separates from the echinid ova studied—except those of *Arbacia*—the cortical break-down, which is responsible for membrane separation, is easily visible under low power of the microscope. Progressively with the separation of the membrane, vesicles appear in the perivitelline space. They gradually disappear as they move across the space from the vitellus toward the membrane. I have repeatedly demonstrated this phenomenon as it occurs in eggs of *Echinarachnius*; many workers, however, though they have observed it have been sceptical of its being a normal process. It was interesting to find that the Naples echinid ova exhibited the same phenomenon.

Concerning the rôle of perivisceral fluid as an inhibitor for fertilization, I need say very little. Here again, Fol pointed out this fact. Other data indicate that such "blood" inhibition is by no means confined to the echinoderms.

Similarly, since some of the earlier work on the agglutination of spermatozoa by specific egg-sea-water was done on European echinids, this topic may be dismissed with a word. My observations at Naples got a long way toward strengthening the position that fertilization capacity and sperm agglutinin production run parallel. Stale eggs from lots which previously had given the optimum fertilization-reaction either gave low or no response to insemination. The evidence from the observations makes it highly improbable that such eggs failed to fertilize because thev were dead.

It would be hazardous to assert that the fertilizin theory based on the reactions between spermatozoa and ova and between spermatozoa and egg-sea-water with and without perivisceral fluid of the few forms studied is capable of application to fertilization in all metazoa. For these particular forms, however, the theory still holds.

LITERATURE CITED.

Just, E. E.

'19 The Fertilization Reaction in *Echinarachnius parma.* I. Cortical Response of the Egg to Insemination. BIOL. BULL., 36, 1–10.

Just, E. E.

'22 Initiation of Development in the Egg of *Arbacia.* III. The Effect of *Arbacia* Blood on the Fertilization Reaction. *Ibid.*, 43, 411–422.

Just, E. E.

'23 The Fertilization Reaction in *Echinarachnius parma.* VII. The Inhibitory Action of the Blood. *Ibid.*, 44, 10–16.

Lillie, F. R.

'14 Studies of Fertilization. VI. The Mechanism of Fertilization in *Arbacia.* Jour. Exp. Zool., 16, pp. 523–590.

Runnström, J.

'24 Zur Kenntnis der Zustandsveränderungen der Plasmakolloide bei der Reifung, Befruchtung und Teilung des Seeigeleies. Acta Zoologica, 5.

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BIOLOGICAL BULLETIN

VARIATION OF NORMAL GERM CELLS. STUDIES IN AGGLUTINATION.

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It has been generally assumed that eggs or sperm, freshly shed from different echinid individuals at the height of the breeding season, are in essentially the same physiologic condition. This assumption has led to gross errors, discordant experimental results and unnecessary confusion.

Nearly everyone who has made prolonged experimental studies with echinoderm .eggs or sperm has recorded evidences of a disturbing variability, even in freshly shed germ cells. There has been, however, little or no agreement concerning (1) the exact physiologic condition at the time of maturing or shedding, (2) the extent of the change or changes at fertilization; whether these changes are in one direction or cyclical, (3) the effect of such changes upon the behavior of the fertilized eggs, (4) the number and kind of changes, and (5) the underlying causes of these changes.

In respect to sperm it has also been assumed that they are relatively constant when shed.

As early as 1883 Born noted that the eggs in the last part of the egg string (*Bufo cinereus*) developed irregularly or did not develop at all. Koehler (1915) made a similar observation for echinid eggs, *i.e.*, eggs were not in the same physiologic state when shed, for those nearest the exit pores were the oldest. Lillie, R. S. (1908, 1915) observed that the age of the eggs (starfish) was an important factor in their subsequent behavior. He corroborated Gemmill (1900) that the eggs changed with age, improving at first and subsequently deteriorating. Morgan [1] noted that the eggs (*S. purpuratus*) showed signs of ageing even when in the ovary, for the jelly layer was reduced as in eggs that were aged for several days in the laboratory.

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[1] Reviewed in his "Experimental Embryology" (1927).

Marked changes have been noted towards the close of the breeding season. Gemmill (1900) among others noted that at this time the echinid eggs lived a shorter time, polyspermy occurred more and more frequently, fertilization was delayed, and sperm lived a shorter time. Heilbrunn (1915) recorded that the concentration of sperm (*Arbacia*) required to elevate the fertilization membrane was greater towards the close of the breeding season, and that there was progressively greater difficulty of the eggs to elevate the membrane. Hyman (1923) also noted changes in the viscosity of ageing eggs.

Lillie, F. R. (1914) concluded that "the condition of the gonads is the most variable thing in summer sea urchins . . ." and in 1915 concluded that the large variations (*Arbacia*) were due to the use of germ cells after the height of the breeding season that variations prior to this time are due to faulty technique, for with proper precautions "both eggs and sperm are relatively constant."

Goldforb (1917, 1918) studied the variability of eggs of three species of echinoderms, *Toxopneustes*, *Hippanoë*, *Arbacia*, studied the variation in size, color, shape, amount of jelly, rate and manner of membrane formation, rate and numbers of fertilized eggs, rate and regularity of cleavage. These studies were made during three seasons. He concluded that freshly shed eggs at the height of the breeding season were not constant, that they showed wide ranges of variability, and sometimes even extreme states of deterioration. He concluded that varying degrees of overripening occurred prior to shedding.

Just (1919) came to a similar conclusion after studying *Echinarachnius* eggs. He records a wide variation due largely to corresponding degrees of deterioration. He also concluded that "ovary" eggs (of *Asterias* also) were more variable and inferior to shed eggs, due to underripeness. Graves (1928) found that *Cummingia* eggs behaved as echinid eggs described by Goldforb, namely, that deterioration occurred before spawning, with a corresponding wide range in the vitality of the eggs. Gee (1916) for *Fundulus* eggs, Calkins (1920) for *Uroleptus*, attributed variations to corresponding internal differences.

Other evidence of changes in the egg, prior to shedding, is afforded by the work of Hertwig (1906, 1907), Kuschekewitch (1910), and Riddle (1914), that overripeness of the eggs decreased developmental energy, and increased the proportion of surviving males. Koehler (1915) noted the tendency of overripe germ cells (echinid) to produce more matroclinous larvæ. Hertwig (1890), Grief (1901), Newman (1921), and others attributed the increase in natural parthenogenesis to ageing of eggs. Fuchs (1914) and East (1915, 1917, 1918) recorded the increase in self fertilization with overripeness of the germ cells.

These citations may serve to indicate the background for the present study of the variation of germ cells, and of the subsequent studies of ageing germ cells.

The present study gives evidence of a surprisingly wide range of variation, not only among eggs, but also among sperm, when these germ cells were freshly collected, freshly shed, and freshly tested, at the height of the breeding season. *These observations emphasize the need, in experimental work, of determining by simple tests, the exact physiologic condition of the eggs and of the sperm of each individual.* By selecting germ cells in approximately the same physiologic condition, one may minimize the disconcerting differences in experimental results.

The experiments were performed in 1924 and in 1926 at the Marine Biological Laboratory at Woods Hole. My thanks are due the Directors for the facilities of the Laboratory.

Technique.

Because so much depends upon the exact details of technique, and to avoid repetition in subsequent studies, a concise statement is here given of the experimental procedure. This is primarily the procedure of Lillie, F. R. (1914). *Arbacia punctulata were brought directly from the collecting boat to the laboratory and immediately tested.* Eggs that spontaneously flowed from the aboral openings are called "shed" eggs. When ovaries were removed as intactly and gently as possible, the eggs thus liberated are termed "ovary eggs." [1] Similarly "shed" and "testes" dry sperm were obtained. The germ cells from each individual were kept separately. Eggs were washed in 300 cc. or more sterile sea-water, and the final egg to water volume was usually 1 to 3. The eggs were gently shaken every ten minutes for one hour. The supernatant or "egg water" was removed, *examined for unripe, ripe, and overripe eggs, and for jelly. If present, they were immediately removed.* Their presence would alter the subsequent agglutination reaction. The "egg water," the solutions therefrom, and the dry sperm, were kept under wet cloths to minimize evaporation and to maintain a lower temperature, 20° C.[2] Capillary mouth pipettes were sterilized (against sperm and agglutinins) in a jar of 8,000 cc. tap water, then rinsed in 3,000 cc. sterile sea-water, then used in transferring the "egg water" solution to the sperm suspension on the slide. Each test was made with a different pipette.

After all the "egg water" solutions were made, each with a different pipette, a 1 per cent. sperm suspension was prepared, two measured drops placed on the slide, and the "egg water" solution gently blown under the raised cover slip. The reversible agglutination of the sperm was measured in seconds. A "loud timer" aided in this.

Sea water was carefully collected in glass at the incoming high tide, filtered, and stored in glass for 3 to 10 days before using, and evaporation minimized, by covering with wet cloths. This sterile sea water was used in all experiments at approximately 20° C.

EXPERIMENTAL ERROR.

The accuracy of the technique was determined in two ways:

1. *Repetition Test.*—The agglutination test was immediately repeated with other samples of the same solution.

2. *Aliquot Part Test.*—Eggs were divided into two equal parts in similar volumes of sea water and similar egg to water dilution. The egg water of each was then tested, one immediately after the other.

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[1] The term "ovary eggs" is used by Just (1919) and myself differently, with correspondingly different results. Just cut the ovaries into pieces and presumably liberated many unripe eggs. In my experiments the ovaries are barely injured, with liberation of minimal number of unripe eggs.

[2] Temp. 20°–22° C. in different experiments.

These two tests were made in nearly every experiment. Any variation in these tests was considered a measure of the experimental error.

Out of 19 such tests the agglutination time was exactly the same in 8 tests. In 6 tests there was a difference of but 1 second. In 1 test the difference was 3 seconds and in 1 test the difference was 4 seconds. *The average difference was 1 second, or 4.5 per cent.* This may be considered the experimental error.

Variation in eggs and in sperm from different individuals was then studied.

THE VARIATION IN FRESHLY SHED EGGS FROM DIFFERENT INDIVIDUALS.

Table I gives the observations in 19 experiments, including 58 females tested separately, 2 to 4 in each experiment. These experiments were made from July 1 to 26. In some experiments the egg to water ratios are not the same for all the females. Hence these ratios are given in each instance so that corrections may be made. A fresh sperm suspension was made for testing the egg waters of two to four females of each series. The table gives the observed agglutination time for each egg water, the calculated agglutination time,[1] the difference or variation in seconds and in per cent.

The freshly collected, freshly shed, and freshly tested eggs in each series varied from 2 to 55 seconds calculated time. The variation was least in Experiments 12, 20 and 26, where the different females differed by 2, 2, and 4 seconds respectively. This is within the experimental error.[2] In 10 experiments the variation was 5 to 10 seconds; in 4 experiments the variation was 11 to 20 seconds; in 1 experiment the variation was 30 seconds, and in 1 experiment the variation was 55 seconds. The average variation was 12.0 seconds. The variation in control experiments ranged from 0 to 4 seconds only, with an average of only 1 second.

The minimal variation was 9 per cent. in 1 experiment. In 3 experiments the variation ranged from 16 to 26 per cent., in 4 experiments from 37 to 45 per cent., in 6 experiments from 66 to 87 per cent., in 3 experiments from 100 to 120 per cent., in 1 experiment 400 per cent., and in 1 experiment 1,300 percent. These averaged 142 per cent. The expermental error averaged 4.5 per cent. *The "best" eggs in each day's collecting at the height of the breeding season varied by as much as 1,300 per cent.*

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[1] Calculated for differences in egg to water ratio.

[2] The percentage differences, 9, 19 and 26 per cent., are however much greater than the maximal experimental error.

TABLE I.

SHOWING WIDE VARIATION IN AGGLUTINATION TIME WHEN "NORMAL" EGGS FROM FRESHLY COLLECTED, FRESHLY EXAMINED FEMALES ARE TESTED BY THE SAME SPERM SUSPENSION.

Exp. No.[1]	♀ No.	Agglutination Time in Sec.	Egg to Sea-water Ratio.	Egg Water Dilution.	Difference in		[2] Calculated Difference in	
					Seconds.	Per cent.	Seconds.	Per cent.
1	1	55	1 : 3	1/100				
	2	27	1 : 3					
	3	25	1 : 3					
	4	25	1 : 3		30	120		
2	1	12	1 : 4	1/60				
	2	35	1 : 8				55	400
3	1	9	1 : 5	1/60				
	2	12	1 : 6					
	3	23	1 : 2				9	100
4	1	9	1 : 5	1/120				
	2	27	1 : 2				10	110
5	1	13	1 : 8	1/160				
	2	15	1 : 8					
	3	8	1 : 8					
	4	23	1 : 4				7	87
6	1	17	1 : 3	1/80				
	2	13	1 : 3					
	3	22	1 : 3		9	69		
8	1	24	1 : 3	1/160				
	2	14	1 : 3					
	3	23	1 : 3		10	71		
10A	1	0	1 : 6	1/320				
	2	13	1 : 6					
	3	10	1 : 6		13	1,300		

Average experimental error—1 second or 4.5 per cent.

Average variation in "normal" fresh eggs—12.0 seconds or 142 per cent.

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[1] These numbers are also the dates in July when each experiment was performed.

[2] To correct for differences in egg to water ratio.

TABLE I.–(Continued)

Exp. No.[1]	♀ No.	Agglutination Time in Sec.	Egg to Sea-water Ratio.	Egg Water Dilution.	Difference in		[2] Calculated Difference in	
					Seconds.	Per cent.	Seconds.	Per cent.
10B	1	23	1 : 3	1/80				
	2	26	1 : 3					
	3	20	1 : 5					
	4	19	1 : 3				7	37
11	1	13	1 : 3	1/320				
	2	16	1 : 3					
	3	14	1 : 5					
	4	15	1 : 3				6	45
12	1	12	1 : 6	1/320				
	2	13	1 : 6					
	3	11	1 : 6					
	4	15	1 : 4				2	19
14	1	29	1 : 2	1/320				
	2	21	1 : 2		8	39		
19	1	50	1 : 3	1/320				
	2	30	1 : 3		20	66		
20	1	23	1 : 5	1/320				
	2	30	1 : 3				2	9
22	1	21	1 : 3	1/300				
	2	26	1 : 3					
	3	18	1 : 3		8	44		
23	1	19	1 : 3	1/300				
	2	18	1 : 2					
	3	19	1 : 3				6	16
24	1	17	1 : 3	1/300				
	2	29	1 : 3					
	3	28	1 : 3		12	70		
25	1	15	1 : 3	1/300				
	2	16	1 : 3					
	3	22	1 : 4					
	4	16	1 : 3				11	77
26	1	13	1 : 2	1/300				
	2	15	1 : 3					
	3	10	1 : 5				4	26

Examination in detail of a few examples may make more clear this extraordinary range in variability. In Experiment I the agglutination was 25 seconds for each of 2 females, 27 seconds and 55 seconds for the other two females with the same egg to water volumes, the same dilution of egg waters, the same sperm suspension. Lillie (1914) showed that a more exact procedure consists in finding a dilution of egg water in which the agglutination time is about 8 seconds. When higher concentrations of egg water are used, the agglutination values are not so exact. This is admitted. The necessity for rapid tests with the different suspensions and solutions necessitated the use of a low concentration of egg water but not necessarily the lowest that would give an 8 second agglutination. The fact that the same concentrations were used throughout makes the results comparable, and the error too small to materially affect the results.

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[1] These numbers are also the dates in July when each experiment was performed.

[2] To correct for differences in egg to water ratio.

In Experiment 8 the agglutination time for the eggs of the 3 females was 24, 14, and 23 seconds respectively. In Experiment 10*A* one female registered 13 seconds, another 10 seconds, *and a third did not agglutinate at all.* In Experiment 22, the values were 18, 21, and 26 seconds respectively.

It should be recalled that this variation occurred in freshly collected urchins, the "best" of the day's collection, at the height of the breeding season, and that the eggs and sperm were freshly shed, immediately after arrival from the collecting boat. These are "normal" eggs. These should register the minimal variation. They actually register a variation from 2 to 1,300 per cent.

In Experiment 19, with a 1/320 egg water dilution, the agglutination time of the eggs of female 1 was 50 seconds. Under exactly the same circumstances the eggs of female 2 registered only 30 seconds. I do not interpret this to mean *that the eggs of female 2 were in the same degree of ripeness as those of female 1, secreting only 3/5 as much agglutinin as female 1.* My interpretation is that in addition to an uncalculable but relatively small genetic difference in agglutinin production[1] *the large difference is due to the greater overripening of the eggs of female 2 prior to shedding.* The evidence in support of this interpretation will be given later. In Experiment 10*A*, with egg water dilutions the same, female 2 registered 13 second agglutination. These fresh "normal" eggs were by a variety of tests shown to be in relatively poor condition, *i.e.*, overripe when shed. The eggs of female 3 were in still poorer condition, *i.e.*, more overripe, registering only 10 second agglutination. The eggs of equally fresh "normal" eggs of female 1 were extremely deteriorated, giving rise to no agglutination at all.

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[1] Loeb and Chamberlain (1915) measured differences in enzyme content of eggs (*Arbacia*) but whether these differences were genetic, as assumed, or due to ageing, is not clear.

A much larger number of experiments were made than those listed in Table I. The 19 listed are one series representative of the unexpected, consistent and large variation in freshly shed or "normal" eggs of *Arbacia*.

It might be objected that the calculated differences, to correct for differences in egg to water ratios, are only approximate. But in the experiments where no such calculation was necessary, because all egg to water ratios were the same, the variation was practically as large, namely, 6, 6, 19, 23, 30, 37, 39, 44, 66, 69, 70, 71, 110, 130 per cent.

This extraordinarily large variation in agglutination time closely corresponds with equally large variations in size, color, shape of eggs, thickness of jelly layer, fertilizability, rate of membrane formation, rate, regularity, and per cent. of cleavage. These latter variations have been shown (Goldforb, '18*a*, 18*b*) to represent corresponding degrees of ageing or deterioration. And it is presumptive and later will be demonstrated in detail that the differences in agglutination time also measure degrees of deterioration.

VARIATION IN FRESHLY SHED SPERM FROM DIFFERENT INDIVIDUALS.

It is concluded that at the height of the breeding season (1) the egg is the actively changing cell, secreting varying amounts of agglutinin; (2) that agglutination time is proportional to the quantity of agglutinin thus liberated. The question arose whether the sperm is a biologic constant, or varies as the egg does.

The following experiments (with those in subsequent studies) demonstrate that *freshly shed sperm from different males are just as widely variable as freshly shed eggs.*

Some typical experiments are brought together in Table II. In Experiment 9, for example, samples of the egg water of female 1 were tested separately by freshly prepared sperm suspensions of three males. The agglutination was 10, 0, and 9 seconds respectively. When fresh suspensions of the same three males were tested with the egg water of female 2, the results were quite different, namely 17, 0, and 13 seconds, respectively. The 0 denotes no agglutination. Fresh suspensions from the same three males against egg water from female 3 gave even larger agglutination values, namely, 23, 0, and 13 seconds respectively.

Table II.

Showing Wide Variation in Agglutination Time When "Normal" *i.e.* Freshly Shed Sperm from Different Males are Tested by the Same Egg Water.

Exp. No.[1]	♀ No.	♂ 1	♂ 2	♂ 3	♂ 4	Difference in	
						Seconds.	Per cent.
9	1	10	0	9		10	1,000
	2	17	0	13		17	1,700
	3	23	0	13		23	2,300
Aver.		17	0	11			
10	1	15	33			18	120
	2	7	22			15	214
	3	12	17			5	41
13	1	15	14	12		3	25
	2	13	8	12		5	62
	3	13	13	10		3	30
	4	13	11	18		8	63
11	1	12	0	12	9	12	1,200
	2	11	0	16	15	16	1,600
	3	25	0	33	9	33	3,300
	4	10	6	18	15	12	200
	5	17	0	22	13	22	2,200
Aver.		15.0	1.2	20.2	12.2		

[1] These numbers are also the dates in July when each experiment was performed.

It should be noted that the *sperm of male 1 gave consistently the longest agglutinations, with all three females. Male 3 gave intermediate values. Male 2, though freshly shed and "normal", did not agglutinate in any egg water.* The average agglutination time for male 1 was 17 seconds, for male 3, 11 seconds, for male 2, none. Male 1 gave 54 per cent. longer agglutination reactions than male 3, and 1,700 per cent. longer than male 2. If male 2, whose sperm were not agglutinated in any of the tests, be omitted, the variation is 1, 4, and 10 seconds, or 11, 30, and 77 per cent.

It is also possible by these tests to pick out which eggs are most potent. Female 3 gave the longest agglutination reactions. Female 1 gave the briefest and female 2 intermediate values.

A given sperm suspension gave different values with eggs from different females. Likewise eggs from one female gave different values with sperm from other males. But eggs or sperm of a given individual gave the same relative values with other germ cells.

In Experiment No. 9 an extraordinarily wide difference in agglutinability occurred in the sperms of the three males. *The variation is as great as among eggs from different females. Agglutination time appears to be dependent upon the physiologic condition of the eggs as well as upon the condition of the sperm at the time of testing.* The next study will consider this in detail.

Other experiments gave essentially similar results. In Experiment 10, 2 males were tested separately against 3 females. Male 2 gave consistently longer agglutinations than male 3, namely, 41, 120, and 214 per cent. respectively. In Experiment 13 the sperm of 3 males were tested separately against the eggs from 4 females. The agglutination time varied by 25, 30, 62, and 63 per cent. respectively. In Experiment No. 11, 4 males were tested against 5 females. Male 3 gave consistently longest agglutination values, with an average of 20.2 seconds. Male 1 averaged 15.0 seconds, male 4 averaged 12.2 seconds, and male 2 gave an average of only 1.2 seconds. This male gave no agglutination in 4 out of 5 females. If male 2 be ignored, the difference in agglutinability of these males was 33, 45, 69, 80, and 266 per cent. If male 2 be included, the differences were 1,200, 1,600, 3,300, 200, and 2,200 per cent. The dry sperm of these 4 males had been recorded at the time of shedding as follows: No. 3 best, No. 1 good, No. 4 poor. Male 2 was not recorded. This is in very close agreement with their agglutinability.

The results are unmistakable. The sperm that gives high agglutination values with 1 female tends to give high, though not the same, values with other females. Vice versa, sperm that gives low agglutination values with one female gives consistently low but not necessarily the same values with other females. The differences with a given sperm are due primarily to differences in the physiologic condition of the eggs of the different females. The reverse is also true, *i.e.*, when a given egg water is tested by different males, the differences observed denote primarily differences in the physiologic condition of the different sperms.

It is evident that the *agglutination time of freshly shed "normal" germ cells is dependent not only upon the condition of the eggs but also upon the condition or agglutinability of the sperm.* Not only do freshly shed eggs from different individuals vary very widely in their ability to agglutinate a given sperm, but a given freshly shed sperm varies as widely in agglutinability, with eggs from different females.

The cause or causes of this variability in sperm will be discussed in the next study.

Variability of Shed Versus Ovary Eggs.

It seemed worth while to compare the agglutination time of "shed" versus "ovary" eggs. It should be recalled that these "ovary eggs" come from females whose *ovaries were removed as intactly and as gently as possible.* These ovary eggs include the minimum of unripe eggs. Such ovary eggs will differ therefore from those described by Just (1919) in which ovaries were cut into pieces, thus liberating many unripe eggs.

For purposes of comparison all experiments, in which the same egg water concentration was used, are brought together in Table III. The eggs of all females in an experiment were tested by the same sperm in fresh suspensions.

Table III indicates that "ovary" eggs tended to give longer agglutination values and a wider range of variability than shed eggs. In the 8 experiments involving 22 females, the "ovary" eggs in each series varied by 1, 4, 5, 7, 8, 8, 12, and 20 seconds respectively. The average was 8.1 seconds. In the 6 experiments including 20 females, the "shed" eggs in each series varied by 1, 1, 3, 4, 6, and 13 seconds. The average was 4.6 seconds. (If female 1 in Experiment No. 9 be omitted, because no agglutination occurred, the variation among the "shed" eggs would be 1, 1, 3, 3, 4, and 6 seconds, or 3 seconds average.) Experiment 2 is especially interesting because the shed and the ovary eggs were tested by samples of the same sperm. The shed eggs gave 11 and 12 seconds respectively, while the ovary eggs in the same egg water concentration gave much longer values, *viz.*, 15 and 35 seconds respectively.

TABLE III.

COMPARISON OF "SHED" AND "OVARY" EGGS IN 1/320 EGG WATER DILUTION. EACH EXPERIMENT TESTED BY THE SAME SPERM SUSPENSION. FIGURES DENOTE AGGLUTINATION TIME IN SECONDS.

Exp. No.[1]	Shed Eggs.				Variation in Sec.	Aver. Aggl. Time.	Ovary Eggs.				Variation in Sec.	Aver. Aggl. Time.
	♀ 1	♀ 2	♀ 3	♀ 4			♀ 1	♀ 2	♀ 3	♀ 4		
2	11	12			1	11.5	15	35			20	25
5							8	9	6	13	5	9
9	0	13	10		13	7.7						
10	12	13	11	15	4	12.7						
11	13	16	14	15	3	14.5						
12	14	9	15	12	6	12.5						
14							29	21			8	25
19							28	27			1	27.5
20							23	30			7	26.5
22							22	26	18		8	22
23	20	19	20		1	14.7						
24							17	29	29		12	25
25							16	16	22	16	4	17.5
Average					4.6	13.2					8.1	20.7

[1] Numbers correspond with dates in July when experiment was performed.

The larger variability among "ovary" than among "shed" eggs is attributed to the presence of a larger proportion of overripe eggs. This will be discussed in the next study. More intensive study needs to be made of "ovary" eggs, with a definite knowledge of the relative numbers of unripe, ripe, and overripe eggs, and the degree of overripeness.

SEASONAL VARIATION.

In the first half of July, 1926, nearly all the *Arbacia* gave numerous shed eggs. In the second half of the month there were very much fewer shed eggs and more "ovary" eggs. The same observations were made in July, 1924. The eggs in the first half of the month were mostly "good" ones, *i.e.*, in good physiologic condition, while those in the second half tended to be "poorer" eggs. There appears to take place two cycles of egg maturing; the first reaches its peak about the middle of July, the second, I am informed, in late August or early September.

It would be of much interest to determine the exact physiologic condition of the eggs and of the sperm at the moment of natural shedding, throughout the breeding season. Vernon's observations ('99) need to be checked by more refined quantitative methods.

Between the first half of July and late August there appears to be a period during which the mature eggs deteriorate rapidly, and few if any unripe eggs mature. The behavior of the eggs in this latter period depends upon the relative numbers of unripe, ripe, and overripe eggs and the degree of overripeness. As these factors seem not to have been taken into account (Gemmill, '00) much confusion has resulted.

The agglutination test is in accord with other tests, all of which force one to conclude that "ovary" eggs show all gradations to extremely overripe eggs.

Other Sources of Variability.

When a comparison was made between the eggs tested immediately after receiving the *Arbacia* from the collecting boat, and eggs from the same group of *Arbacia* kept in tanks of running sea water 3 or more days, the eggs from the latter were more deteriorated. Even when the *Arbacia* were kept several days in the large floats at the wharf in the harbor, the eggs were more deteriorated than those eggs freshly tested upon receipt from the collecting boats. When the *Arbacia* were exposed to the sun during the trip to the laboratory, *i.e.*, when large numbers were kept in pails with insufficient sea water or exposed to the sun, such *Arbacia* gave a preponderance of deteriorated eggs.

When individual *Arbacia* were kept in jars containing 1,000 cc. to two gallons of sea water, changed twice daily, and the jars placed in running sea water, the germ cells spontaneously shed from the intact animals. These germ cells were tested. The tests were size, color, shape of egg, rate of membrane formation, rate of cleavage and agglutination time. Many such spontaneously shed germ cells were in excellent physiologic condition. *Yet not infrequently such eggs showed surprising degrees of degeneration.* Such degenerate eggs were shed late, while the less degenerate eggs were shed early in the egg cycle. I attribute such degeneration to delayed shedding of eggs.

It was also noted that for several days after severe and protracted storms, *Arbacia* freshly collected and freshly tested gave germ cells in a deteriorated condition. It would seem as though severe storms or other adverse condition delays the natural shedding of germ cells with a consequent degeneration, the extent of degeneration being a function of the time that ripe eggs are retained within the body, and a function of the temperature of the sea water.

Extrusion of eggs, whether spontaneously or after opening the body, gives no assurance that the eggs are recently matured, *i.e.*, in good condition.

My evidence for "shed" versus "testes" sperm is not sufficient to draw any definite conclusions.

SUMMARY.

1. *Arbacia* were freshly collected, freshly opened, the "best" eggs selected and immediately tested for agglutination time. The technique gave for duplicate tests, or for tests of aliquot portions of eggs, a difference in agglutination time of 0 to 4 seconds with an average difference of 1 second or 4.5 per cent. This is the experimental error.

2. (*a*) Eggs from different females when tested separately, under strictly comparable conditions, by the same sperm suspension from a single male, gave extremely wide differences in agglutination time, namely, 2 to 55 seconds or 9 to 1,300 per cent. Eggs that gave high agglutination values with one sperm gave consistently high, though not the same values with sperm from other males.

(*b*) These variations in agglutination time corresponded with the variations in size, color and shape of eggs, loss of jelly, rate and per cent. of membrane formation, rate and per cent. of cleavage. All of them measure degrees of deterioration or overripening prior to shedding. Hence agglutination time may be used as another quantitative measure of deterioration of eggs.

(*c*) "Shed" eggs gave lower agglutination values and are less variable than "ovary" eggs, as defined, due to larger number of overripe eggs in the "ovary eggs."

(*d*) Severe storms and other adverse conditions that delay spontaneous shedding tend to deteriorate the eggs within the body, with corresponding changes in agglutination values.

3. (*a*) Suspensions of sperm from different males, in the same concentration, tested with the same egg water also gave a surprising amount of variation. They varied from 11 to 3,300 per cent.

(*b*) Sperm which gave high agglutination values with the eggs of one female gave consistently high, though not the same, values with eggs of other females. Sperm with low agglutination values gave low values with other females.

4. These large differences in agglutinability of different freshly shed sperms are due to corresponding physiologic deterioration or overripening prior to shedding.

5. The large differences in freshly shed eggs from different females is in small part due to genetic differences in agglutinin production, in largest part to deterioration of eggs within the body, prior to shedding.

6. Chronologically fresh, *i.e.*, "normal" germ cells may range from physiologically fresh to extremely overripe germ cells.

BIBLIOGRAPHY.

Born, G. Arch. Ges. Physiol., 1883, **32**.
Calkins, G. J. Exp. Zool., 1920, **31**.
Child, C. M. Arch. f. Entw., 1923, **32**.
East, E. M. Amer. Nat., 1915, **49**.
East, E. M. Proc. Am. Phil. Soc., 1915, **54**.
East, E. M., and Park, J. B. Genetics, 1917, **2**.
East, E. M., and Park, J. B. Genetics, 1918, **3**.
East, E. M., and Mangelsdorf, A. J. Proc. Nat. Acad. Sc., 1925, **11**.
Fuchs, H. M. Arch. f. Entw., 1914, **40**.
Fuchs, H. M. J. Genetics, 1914, **4**.

Gee, W. Biol. Bull., 1916, 31.
Gemmill, J. J. J. Anat. and Physiol., 1900, 34.
Goldforb, A. J. Proc. Nat. Acad. Sc., 1917, 3.
Goldforb, A. J. Anat. Record, 1917, 11.
Goldforb, A. J. Carnegie Institution 1917, Publication No. 251.
Goldforb, A. J. Biol. Bull., 1918, 34.
Goldforb, A. J. Biol. Bull., 1918, 35.
Grave, B. H. Biol. Bull., 1928, 54.
Heilbrunn, L. V. Biol. Bull., 1915, 29.
Hertwig, O. and R. Jena Zeit. f. Naturw., 1886, 19.
Hertwig, O. Handb. d. vergl. w. exp. Entw. Ges., 1906.
Hertwig, R. Deutsche Zool. Gesells. Verhandlung, 1907, 17.
Hyman, L. Biol. Bull., 1923, 45.
Just, E. E. Biol. Bull., 1919, 36.
Just, E. E. Biol. Bull., 1919, 36.
Koehler, O. Arch. Zellf., 1912, 8.
Koehler, O. Ber. Ges. Freiburg, 1914, 20.
Koehler, O. Zeit. Ind. Abst. u. Vererb., 1915, 15.
Kuschakewitsch, S. Festschr. f. R. Hertwig, 1910.
Lillie, F. R. J. Exp. Zoöl., 1913, 14.
Lillie, F. R. J. Exp. Zoöl., 1914, 16.
Lillie, F. R. Biol. Bull., 1915, 28.
Lillie, R. S. J. Exp. Zoöl., 1908, 5.
Loeb, J., and Chamberlain, M. M. J. Exp. Zoöl., 1915, 19.
Morgan, T. H. Experimental Embryology, 1927.
Newman, H. J. Exp. Zoöl., 1921, 33.
Newman, H. Biol. Bull., 1917, 32.
Riddle, O. Bull. Amer. Acad. Med., 1914, 15.
Vernon, H. M. Proc. Royal Soc. B., 1899, 65.
Vernon, H. M. Arch. f. Entw., 1900, 9.

CHANGES IN AGGLUTINATION OF AGEING GERM CELLS.

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Previous studies (Goldforb, '17*a*, '17*b*, '18*a*, '18*b*, '29) led to the conclusion that freshly shed eggs, from freshly collected individuals, vary widely in a number of morphologic and physiologic traits, such as size, color, shape of eggs, amount of jelly, duration of agglutination, rate and manner of membrane formation, rate and percentage of segmentation, etc. Freshly shed sperm from different males, freshly collected and freshly prepared, were just as variable in their agglutinability, fertilizability, etc.

This wide range of variability, in all these traits, of so-called "normal" germ cells represented to a minor degree genetic differences, and to a major degree differences in physiologic condition. In other words, during the breeding season the freshly shed germ cells may be freshly matured or in varying and marked degrees of overripeness. When the germ cells were aged outside of the body, no new changes occurred, but merely a continuation or intensification of the morphologic and physiologic changes begun within the body of the sea urchin.

The present study [1] was undertaken to throw further light on the ageing phenomena with particular reference to agglutination, and to correlate the agglutination changes with those previously studied in ageing germ cells.

Technique.

For a description of the technique and for evidence of its adequacy I refer to the previous study (Goldforb, '29). It is important to note that the *Arbacia* were freshly collected, the germ cells freshly shed, the eggs washed in 300 cc. of "sterile" sea water collected at high tide. The ratio of eggs to sea water was usually 1 to 3.[1] The exact ratios are given in the Tables. At the end of one hour a sample of the supernatant egg water was taken, and, if any eggs or jelly were present, they were immediately removed. The egg water was then diluted, and this solution used throughout the experiment. At each age, thereafter, the same procedure was used with the eggs of each female. For each test a 1 per cent. sperm suspension was prepared from a freshly opened male. The results are therefore strictly comparable. The experimental error was 1 second or 4.5 per cent. (Goldforb, '29).

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[1] The experiments were performed at the Marine Biological Laboratory at Woods Hole in 1924 and 1926. I wish to acknowledge my thanks to the Directors for the facilities of the Laboratory.

I.

AGGLUTINATION WHEN EGGS ONLY WERE AGED.

Eggs in Good Condition When Shed.

This section includes experiments in which ageing eggs were tested, at each successive interval, with freshly prepared 1 per cent. suspensions of freshly shed dry sperm. The results are brought together in Tables II*A* and II*B*. These Tables give the age, or time after shedding of the eggs and sperm, the egg to water ratio at each age for each female, the agglutination in seconds, the difference in agglutination time between the initial and each subsequent age, the calculated difference in agglutination time, when the egg to water ratios were not the same.[2] The tests were made in different dilutions of egg water, but only one is given in the Tables. The other dilutions gave similar results. Fifty-nine tests are recorded.

The results are summarized in Table I; the individual experiments are given in Table II.

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[1] Even when egg to water ratios were the same, and the solutions, made at successive intervals, were in the same concentration, the resulting agglutinin concentration was not necessarily the same. For as eggs become increasingly overripe they lose their jelly layer with corresponding increase of egg mass to water. This alteration of the ratio is in part compensated by progressive enlargement of overripening eggs. There appears to be no feasible way of calculating these changes. Fortunately the differences in agglutinin production and reactibility of sperm, due to ageing, are so much greater than the error here indicated that the agglutination phenomenon is not seriously affected.

[2] These calculated values are approximate. It should however be noted that these approximations are in very close agreement with the results obtained when the egg to water ratios were the same at successive ages.

It should be recalled that when the eggs were in good physiologic condition at the time of shedding, and aged at approximately 20° C., under the described conditions, there is no disintegration during the first 24 hours. After 24, and usually after 36 or 48 hours, disintegration may begin with the liberation of anti-agglutinins with a corresponding reduction in agglutination values. Any decrease in values during the first 24 hours may not be attributed to disintegration but to decreased liberation of agglutinins. On the other hand, eggs in poor condition when shed, or eggs in good condition but kept in an adverse environment, such as high temperature, may disintegrate during the first 24 hours. The decreased agglutination values of such eggs may be attributed to lowered agglutinin liberation and to the liberation of anti-agglutinins. The exact physiologic condition of the eggs at the time of shedding is determined by the change in size, color, shape, viscosity, amount of jelly, rate and per cent. of fertilization, regularity and per cent. of cleavage, etc. These symptoms showed that the *eggs in Experiments 5,*

TABLE I.

SUMMARY SHOWING CHANGE IN AGGLUTINATION TIME WITH AGEING OF EGGS.

A. Total—All Experiments Combined.

Age of Eggs.	No. of Experiments in Which Agglutination			Per cent. Experiments in Which Agglutination		
	Decreased.	Increased.	No Change.	Decreased.	Increased.	No Change.
3 hrs.	1	8	4	8	61	33
4–9 hrs.	7	14	1	32	63	5
22–25 hrs.	9	11	4	37	46	16

B. Eggs in Good Condition when Shed.

Age of Eggs.	Decreased.	Increased.	No Change.	Decreased.	Increased.	No Change.
3 hrs.	1	6	3	10	60	30
4–8 hrs.	0	12	1	0	92	7
24–25 hrs.	3	9	2	21	64	14

C. Eggs in Poor Condition when Shed.

Age of Eggs.	Decreased.	Increased.	No Change.	Decreased.	Increased.	No Change.
3 hrs.	0	2	1	0	66	33
4–9 hrs.	7	2	0	78	22	0
22–24 hrs.	6	2	2	60	20	20

TABLE II *A*.

SHOWS PROGRESSIVE AND MARKED INCREASE IN AGGLUTINATION TIME WITH AGEING OF EGGS. *Eggs in Good Condition when Shed.* EGG WATER DILUTIONS THE SAME THROUGHOUT EACH EXPERIMENT.

Exp. No.	♀ No.	Age of		Aggl. in Sec.	Egg to Water Ratio.	Age of		Aggl. in Sec.	Egg to Water Ratio.	Observed Change in Sec.	Calc. Value in Sec.	Calc. Change in Sec.	Aver. Change in %.
		Eggs.	Sperm.			Eggs.	Sperm.						
9	1	1 hr.	1 hr.	17	1 : 3	24	1	24	1 : 3	+ 7			+ 50
	2			13	1 : 3			23	1 : 3	+ 10			
	3			22	1 : 3			31	1 : 3	+ 9			
12	1	1	1	12	1 : 6	25	1	12	1 : 8		13	+ 1	62
	2			13	1 : 6			18	1 : 8		19	+ 6	
	3			11	1 : 6			15	1 : 17		30	+ 19	
	4			15	1 : 4			7	1 : 30		21	+ 6	
23	1	1	1	19	1 : 2	3	1	54	1 : 2	+ 35			+ 211
	2			18	1 : 2			75	1 : 2	+ 57			
	3			19	1 : 3			46	1 : 3	+ 27			
						5	1	19	1 : 4		29	+ 10	+ 84
								38	1 : 2	+ 20			
								36	1 : 3	+ 17			
						8	1	27	1 : 7		54	+ 35	+ 132
								24	1 : 5		46	+ 28	
								20	1 : 6		30	+ 11	
						24	1	10	1 : 6		20	+ 1	+ 48
								18	1 : 6		36	+ 18	
								10	1 : 6		15	− 5	
24	1	1	1	17	1 : 3	3	1	16	1 : 4		20	+ 3	+ 5
	2			29	1 : 3			24	1 : 4		34	+ 5	
	3			28	1 : 3			18	1 : 5		24	− 4	
						5	1	17	1 : 6		25	+ 8	+ 56
								30	1 : 6		45	+ 16	
								27	1 : 7		46	+ 26	
25	1	1	1	15	1 : 3	3	1	16	1 : 3	+ 1			+ 22
	2			16	1 : 3			29	1 : 3	+ 13			
	3			22	1 : 4			22	1 : 4	0			
	4			16	1 : 3			16	1 : 3	0			
						6	1	55	1 : 4		60	+ 45	+ 103
								24	1 : 3	+ 8			
								24	1 : 3		22	0	
								34	1 : 3	+ 18			
						24	1	0	1 : 6			− 15	− 4
								14	1 : 5		17	+ 3	
								15	1 : 4	− 7			
								16	1 : 7		32	+ 16	

TABLE II *B*.

SHOWS CHANGE IN AGGLUTINATION TIME WHEN THE *Eggs Were in Poor Condition when Shed.*

Exp. No.	♀ No.	Age of		Aggl. in Sec.	Egg to Water Ratio.	Age of		Aggl. in Sec.	Egg to Water Ratio.	Observed Change in Sec.	Calc. Value in Sec.	Calc. Change in Sec.	Aver. Change in %.
		Eggs.	Sperm.			Eggs.	Sperm.						
5	1	1 hr.	1 hr.	17	1 : 4	22	1	17	1 : 5		19	+ 2	
	2			22	1 : 3			13	1 : 5		18	− 4	
	3			13	1 : 4			14	1 : 10		28	+ 15	− 16
	4			26	1 : 1			0	1 : 4		0	− 26	
20A	1	1	1	29	1 : 2	24	1	21	1 : 2	− 8			
	2			21	1 : 2			20	1 : 3		22	+ 1	
	3			24	1 : 3			26	1 : 5		36	+ 12	+ 6
	4			27	1 : 3			26	1 : 4		28	+ 1	
20B	1	1	1	23	1 : 5	4	0	26	1 : 6		29	+ 6	
	2			27	1 : 3			19	1 : 4		21	− 6	0
						6½	0	18	1 : 6		20	− 3	
								12	1 : 4		14	− 13	− 32
						9	0	22	1 : 5		17	− 6	
								19	1 : 4		21	− 6	− 24
						23	0	20	1 : 5	− 3			
								14	1 : 4		16	− 11	− 28
22	1	1	1	21	1 : 3	3	0	21	1 : 3	0			
	2			26	1 : 3			38	1 : 3	+ 12			
	3			18	1 : 3			23	1 : 3	+ 5			+ 21
						5		11	1 : 8		28	+ 7	
								13	1 : 5		20	− 6	− 6
								9	1 : 5		13	− 5	

20A, 20B, and 22 were in poor physiologic condition when shed, and it is precisely in these experiments that little or no increase in agglutination values occurred with ageing of eggs. On the other hand the eggs in Experiments 9, 12, 23, 24, and 25 were, by these symptoms, in good condition when shed, and *it is in these eggs that a definite, progressive and marked increase in agglutination values took place with ageing of eggs.*

In Table I these two groups are separated. Such separation brings out in sharp relief that the *behavior of ageing eggs depends upon their condition when shed.* Part A includes all experiments. Part B includes the experiments in which the eggs were in good condition when shed. *These eggs showed a material increase in agglutination values in 92 per cent. of the tests.* The increase reached a maximum in 3 to 5 hours. Thereafter there was a slow decrease in values. When 24 hours old, 64 per cent. of the tests were still greater than the initial ones.

On the other hand, eggs in poor condition when shed (Part C) gave increased agglutination values with age, in only 66 per cent. of the tests. The maximum values were reached earlier, *i.e.*, in 0 to 3 hours, and are only slightly above the initial tests or no higher. In 22 to 24 hours 60 per cent. of the agglutination values were below and only 20 per cent. above the initial values. See Figures 1*a* and 1*b*.

The analysis of a few experiments will clarify the results and the conclusions.

In Experiment 23, Table II*A*, the eggs were in good condition at time of shedding. Three females were used. Their eggs were tested separately, at each age, *viz.*, 1, 3, 5, 8, and 24 hours, by freshly shed, freshly prepared sperm suspensions. The initial tests gave agglutination values of 19, 18, and 19 seconds respectively. When 3 hours old, and the egg water dilutions the same, the agglutination values were surprisingly greater, namely, 54, 75, and 46 seconds. *The increase in agglutination values was therefore 35, 57, and 27 seconds, or 284 per cent., 416 per cent., and 242 per cent. respectively.* When these eggs were 5 and 8 hours old the values decreased, yet were much greater than the initial values. The 5 hours old eggs agglutinated 10, 20, and 17 seconds longer than the initial tests. The 8 hour old eggs gave 35, 28, and 11 seconds longer agglutinations than the initial tests. When the eggs were 24 hours old the values dropped sharply; female No. 1 had returned to the initial value, female No. 3 had decreased below, and only female No. 2 was still appreciably above the initial value. In this experiment ageing of eggs gave rise to a very large increase in agglutination. The maximum values occurred about the third hour of ageing, thereafter there was a very slow reduction in values, approximating the initial ones about the twenty-fourth hour.

The results in other experiments are in fundamental agreement with the one just described. The rate and the amount of increase depended primarily upon the degree of overripeness of the eggs at the time of shedding.

In Experiment 25, when the eggs were 3 hours old, only one female increased considerably above initial values, namely 13 seconds. The other 3 females exhibited no change at this age. But when 6 hours old, three of the females gave substantial increases, namely 45, 8, and 18 seconds, respectively, or an average increase of 103 per cent. One female only gave no increase in agglutination values, and this female gave no increase at any age. Reference to my protocol discloses the fact that the eggs of this female were "ovary" eggs, few in number (only 1 cc.)' and by various tests were overripe when shed. These were the only eggs that did not give rise to marked increase in agglutination values with age. With further ageing there was a return towards or below the initial values in all four females. When 24 hours old, two still registered 3 and 16 seconds above, one registered 15 seconds below and one 7 seconds below the initial values.

In Experiment 24, when the eggs were 3 hours old, the change in agglutination was small, namely + 3, + 5, − 4 seconds. When 5 hours old there was a substantial increase in all females, namely + 8, + 16, + 26 seconds respectively, or an average of + 56 per cent.

In Experiments 9 and 12, tests were made when the eggs were one hour and 24 hours old. Intermediate values were not determined. Six out of the 7 females tested gave increased agglutination values *even at the twenty-fourth hour of ageing*. The increases were 7, 9, and 10 seconds for the 3 females of Experiment 9, and 1, 6, 6, 19 seconds for the 4 females of Experiment 10. The average increase was 56 per cent.

Where differences in egg to water ratios necessitated corrections, the results are in substantial agreement with those experiments in which the egg to water ratios were the same throughout the experiment.

Different males were used at each test with the possibility of introducing thereby a source of considerable variation (Goldforb, '29). The results, however, are surprisingly consistent. *Eggs in good condition when shed gave rise to marked and progressive increase in agglutination values during the first 3 to 6 hours of ageing.* After this age there was a slow reduction in values. By the 24th hour some have been reduced to the initial values, some have not yet done so and a few are below initial values.

It is concluded that eggs in good condition liberate increasing quantities of agglutinin during the first 3 to 6 hours, and liberate decreasing quantities thereafter.

Eggs in Poor Condition When Shed.

The behavior of eggs in poor condition when shed is very different. See Table II*B*. In Experiment 20*B*, for example, the agglutination values for the freshly shed eggs were 23 and 27 seconds respectively. When 4 hours old, the eggs of female No. 1 gave a small *increase* of 6 seconds, those of female No. 2 a *decrease* of 6 seconds. When the eggs were 6½ hours old, both females registered values below the initial ones, namely − 3 and − 13 seconds respectively. When 9 and 23 hours old the values were essentially the same as 6½ hour eggs. These eggs, in poor condition when shed, gave with ageing either an exceedingly small and early increase (in one female only), or a progressive decrease. Lillie, F. R., '14, Lillie, F. R., and Just, E. E., '24, Just, E. E., '19, have described this decreasing agglutination phase. The literature does not contain references to the first or increasing agglutination phase, described above.

In Experiment 22, though the eggs were in moderately good condition when shed, they were precociously aged by the high temperature of the laboratory (29° C.) during the greater part of the experiment. These eggs gave in the initial tests 21, 26, and 18 seconds respectively for the 3 females tested. When the eggs were 3 hours old, there occurred an increase of 0, 12, and 5 seconds respectively, an increase of 26 per cent. When 5 hours old, with rising temperature, only female No. 1 gave a small further increase in agglutination. The other 2 females gave values below the initial ones.

In Experiments 5 and 20*A* the eggs were in poor condition when shed. They were tested when 1 and 22 hours old. The old eggs registered an increase in two females, no change in three females, and decreases of 4, 8 and 26 seconds in the other 3 females. The average decrease was 5 per cent.

Taking all the experiments in Table II*B* together it is seen that when the eggs were overripe at the time of shedding, agglutination increased in only 6 instances, and the increases were 5 to 15 seconds. In 6 other instances there was no change or the change was within the experimental error and in 10 instances there was a decrease of 4 to 26 seconds.

Figure 1*b* is markedly different from Figure 1*a*, which gives the results with eggs ripe when shed, and shows the considerable increase in agglutination. Figure 1*b*, for overripe eggs, shows a small or no increase.

I interpret these results to mean that beginning with maturation of the egg, there is a progressive and marked increase in the production or liberation of agglutinin, reaching maximal values not at maturity but in 3 to 6 hours later. Thereafter there is a slow progressive decrease in agglutinin production.

Since eggs from different females are in different stages of ripeness, the rate of agglutinin liberation, at a given temperature and H-ion concentration, etc., depended upon the degree of overripeness of the eggs at the beginning of the experiment, as well as the time or subsequent ageing. If just matured there will be a rapid and marked increase followed by a slow decrease. If slightly overripe when shed, there will be a smaller and earlier increase. If more overripe when shed, there will be no increase, but only decreasing agglutinin production. An increase in temperature or in OH ions accelerates the rate of ageing and hence the rate of the agglutinin cycle. It appears that the total amount of agglutinin is fixed, the more agglutinin liberated prior to the experimental period the less agglutinin is available thereafter.

II.

Agglutination When Sperm Only Are Aged.

The experiments brought together in this section are the reverse of those in the preceding one. A given dry sperm is tested, at each successive age, by freshly prepared egg water solution from freshly shed eggs. These experiments should register the change, if any, in the agglutinability of ageing sperm. The results are brought together in Tables III and IV.

Table III summarizes all experiments. It is evident that *just as in the case of ageing eggs, ageing of sperm is associated with a marked and progressive increase in agglutinability.*

Table III.

Showing Change in Agglutinability of Ageing Sperm.

A. All Experiments Combined.

Age of Sperm.	No. of Experiments in Which Agglutination			Per cent. Experiments in Which Agglutination		
	Decreased.	Increased.	No Change.	Decreased.	Increased.	No Change.
3 hrs.	4	6	5	27	40	32
4–9 hrs.	13	10	6	45	34	20
21–28 hrs.	4	29	0	12	88	0

B. Sperm in Good Condition when Shed.

Age of Sperm.	Decreased.	Increased.	No Change.	Decreased.	Increased.	No Change.
3–4 hrs.	0	5	3	0	63	37
5–9 hrs.	0	10	4	0	71	28
21–28 hrs.	1	29	0	3	97	0

C. Sperm in Poor Condition when Shed.

Age of Sperm.	Decreased.	Increased.	No Change.	Decreased.	Increased.	No Change.
3 hrs.	3	1	2	50	16	33
5–8 hrs.	13	0	2	87	0	13
24 hrs.	3	0	0	100	0	0

Out of 52 tests 40 per cent. gave increased agglutination values when the dry sperm was 3 hours old. Approximately the same

TABLE IV *A*.

CHANGE IN AGGLUTINATION TIME IN AGEING SPERM. *Sperm in Good Condition* WHEN SHED.

Exp. No.	♀ No.	Age of		Aggl. in Sec.	Egg Water Dilution.	Age of		Aggl. in Sec.	Observed Change in Sec.	Calculated Change in Sec.[3]	Average Change in %.	Aggl. Time, Same Egg Water but Freshly Shed Sperm.
		Eggs.	Sperm.			Eggs.	Sperm.					
6	1	1 hr.	1 hr.	17	1/80	1 hr.	24 hr.	40	+ 23		+ 107	
	2			13				28	+ 15			
	3			22				40 +	+ 18 +			
					1/80	1 [1]	24	58	+ 41		+ 204	
								20	+ 7			
								80 +	+ 58 +			
					1/320	1 [2]	28	28		+ 56	+ 354	
								13		+ 26		
								51		+ 102		
					1/320	1 [2]	6	26		+ 52	+ 123	
								13		+ 26		
								19		+ 38		
20B	1	1	1	23	1/320	1	4	25	+ 2		− 6	20
	2			27				22	− 5			21
					1/320	1	6½	23	0		− 2	16
								25	− 2			18
					1/320	1	9	29	+ 6		+ 14	26
								25		+ 1		23
					1/320	1	23	37	+ 14		+ 52	33
								39	+ 12			28
19	1	1	1	29	1/320	1	26	50	+ 21	+ 26	+ 76	26
	2			21				30	+ 9	+ 12		29
10A	1	1	4	0		1	25	15	+ 15		+ 85	12
	2			13				16	+ 3			13
	3			10				14	+ 4			11

[1] Egg water 24 hours old.

[2] Egg water 28 hours old.

[3] Calculated for change in egg water dilution.

TABLE IV *A*.—(*Continued*).

Exp. No.	♀ No.	Age of		Aggl. in Sec.	Egg Water Dilution.	Age of		Aggl. in Sec.	Observed Change in Sec.	Calculated Change in Sec.[3]	Average Change in %.	Aggl. Time, Same Egg Water but Freshly Shed Sperm.
		Eggs.	Sperm.			Eggs.	Sperm.					
						1[1]	25	15	+ 15			
								16	+ 3		+ 85	
								14	+ 4			
26	1	1	1	13	1/300	1	21	15	+ 2			
	2			15				19	+ 4		+ 31	
	3			10				16	+ 6			
24	1	1	1	17	1/320	1	3	24	+ 7			18
	2			29				28	− 1		+ 10	25
	3			28				30	+ 2			26
						1	5	25	+ 8			17
								32	+ 3		+ 17	33
								30	+ 2			23
25[4]	1	1	1[4]	15	1/300	1	3[4]	15	0			11
	2			16				23	+ 7		+ 11	20
	3			22				20	− 2			17
	4			16				19	+ 3			12
					1/300	1	6[4]	75	+ 60			20
								70	+ 54		+ 220	28
								60	+ 38			26
								16	0			20
					1/300	1	24[4]	0				28
								0			− 100	16
								0				13
								0				0

[1] Egg water 24 hours old. [2] Egg water 28 hours old. [3] Calculated for change in egg water dilution. [4] Sperm moderately overripe when shed.

TABLE IV B.

CHANGE IN AGGLUTINATION TIME IN AGEING SPERM. *Sperm in Poor Condition* WHEN SHED.

Exp. No.	♀ No.	Age of		Aggl. in Sec.	Egg Water Dilution.	Age of		Aggl. in Sec.	Observed Change in Sec.	Average Change in %.	Aggl. Time, Same Egg Water but Freshly Shed Sperm.
		Eggs.	Sperm.			Eggs.	Sperm.				
22	1	1 hr.	1 hr.	21	1/300	1 hr.	3 hr.	12	− 9	− 27	19 Temp. 29° C.
	2			26				20	− 6		18
	3			18				15	− 3		13
					1/300	1	5	17	− 4	− 24	
								18	− 8		
								14	− 4		
					1/300	1	8	13	− 8	− 47	
								12	− 14		
								9	− 9		
23	1	1	1	19	1/300	1	3A	21	+ 2	+ 1	19
	2			18				18	0		17
	3			19				18	− 1		16
					1/300	1	3B	28	+ 9	+ 57 [1]	
								30	+ 12		
								—	—		No record
					1/300	1	5	19	0	− 5	
								17	− 1		
								17	− 2		
					1/300	1	6	14	− 5	− 25	
								15	− 3		
								13	− 6		
					1/300	1	8	12	− 7	− 37	
								12	− 6		
								11	− 8		
					1/300	1	24	0	− 19	− 100	
								0	− 18		
								0	− 19		
					1/300	1	19B	40	+ 21	+ 46 [1]	
								24	+ 6		
								18	− 1		

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[1] This sperm not overripe when shed.

per cent. gave increased values when the sperm was 4 to 9 hours old. But 88 per cent. gave increased values when the sperm was 21 to 28 hours old.

The separation of sperm in good physiologic condition at the time of shedding, from those in poor condition, brings out in sharp relief the marked difference in the behavior of these two kinds of sperm. Sperm ripe when shed (Table III*B*), then aged 3 to 4 hours, gave increased agglutination values in 63 per cent. of the tests. The other 37 per cent. registered no change from the initial tests. When the sperm were 5 to 9 hours old, slightly more (71 per cent.) gave increased values. When the sperm were 21 to 28 hours old, practically all of the tests (97 per cent.) gave agglutination values in excess of the initial ones.

On the contrary, sperm overripe when shed (Table III*C*), and then aged for 3 hours, gave increased values in only 16 per cent. of the tests, while 33 per cent. gave no change, and 50 per cent. gave decreased agglutination values. When the sperm was 5 to 8 hours old not a single test gave values greater than the initial ones, and 87 per cent. gave values below the initial ones. When 24 hours old all the tests (100 per cent.) were below the initial values.

In other words sperm ripe when shed agglutinated *increasingly* during the first 24 to 28 hours. How much longer the values might have increased I did not determine. On the other hand, sperm overripe when shed progressively *decreased*. Ninety-seven per cent. of the tests with ripe sperm gave progressively increased values, while 100 per cent. of the tests with overripe sperm gave progressively decreased values.

As the freshly shed eggs, at each interval, were different, and as this introduces an incalculable variation, the eggs of several females were tested at each age. While there is a variation, the increase in agglutination time, for a given batch of eggs, is so much greater than the variation among different batches, that this variant may be ignored.

It should furthermore be recalled that the increase in agglutination time, in these experiments, must be referred to increased agglutinability of sperm and not to increased agglutinin of the eggs.

Sperm in Good Condition When Shed.

Experiment 6 (Table IV*A*) may be taken as an illustration of the behavior of sperm ripe when shed. The freshly shed dry sperm, in freshly prepared 1 per cent. suspension, was tested by freshly shed eggs of 3 females, and agglutinated for 17, 13, and 22 seconds respectively. A second test was made immediately thereafter with samples of the same egg waters, in the same dilution, but with different sperm, kept 24 hours in the dry state.[1] All tests were made with fresh 1 per cent. sperm suspensions. *The 24 hour sperm gave a large and unexpected increase in agglutination values in all 3 females*, namely, 23, 15, and 18 seconds, or 107 per cent. greater than the initial tests. That this large increase was not fortuitous was shown by testing samples of the first egg waters, 24 hours later, with the first male whose sperm was now 24 hours old. The agglutination values were again greatly increased in every test, by 41, 7, and 58 seconds, or 204 per cent. When the sperm was 28 hours old, and tested against samples of the same egg waters, all 3 females registered values greater than when the sperm was 24 hours old. The average increase was now 354 per cent. When the same egg waters were tested by other sperm, 6 hours old, though the increase in values is very large (123 per cent.) it is lower than the 28 or the 24 hour sperm. The increase in agglutination values is correlated with the age of the sperm. While the corrections for differences in egg water dilution are only approximate, there can be no doubt but that old sperm gave consistently and in every test very much greater agglutination values than freshly shed sperm.

In Experiment No. 19, the fresh sperm gave a 29 and a 21 second reaction with the eggs of two females. The next test was made when the dry sperm was 26 hours old, and gave an observed increase of 21 and 9 seconds, a calculated increase of 26 and 12 seconds, or 76 per cent. The same egg waters were immediately tested with a fresh sperm and gave only − 3 and + 8 seconds, or 10 per cent. increase beyond initial values.

Experiments 10*A* and 26 gave similar results. At the end of 21 hours the 3 females in Experiment 26 averaged 31 per cent.

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[1] At 20° C.

greater, and in Experiment 10 at the end of 25 hours they averaged 85 per cent. greater than the initial values. Tests with fresh sperm gave essentially initial values in 2 out of 3 tests.

In Experiment 24 the sperm was tested when 1, 3, and 5 hours old. The 3 hour sperm gave a very small increase (10 per cent.), the 5 hour sperm gave a little larger increase (17 per cent.). The maximum values had presumably not been reached at this age. Tests with fresh sperm gave no increase.

In Experiment 20*B* there was little or no increase in values when the sperm was 4 and 6 hours old. When 9 hours old the increase was 14 per cent. When 23 hours old the increase was 52 per cent. At each age, not only was the sperm tested by freshly shed eggs, but these eggs were again tested by freshly shed sperm. Comparison of these tests emphasizes the conclusion that ageing of dry sperm gave rise to progressively increased agglutination values, while non-aged sperm gave close to the initial values. Figure 3*a* represents this rise in agglutination with ageing of sperm.

Experiment 25 is an example of a different type of experiment, in which sperm were moderately overripe when shed. The agglutination values of the freshly shed germ cells were 15, 16, 22, and 16 seconds for the 4 females tested. When the dry sperm was 3 hours old it was tested with egg waters from the freshly shed eggs of 4 other females. The change in values was exceedingly small, 0, + 7, − 2, + 3 seconds respectively. The average increase was only 11 per cent.

When, however, the sperm was 6 hours old (and tested with freshly shed eggs from 4 other females) the agglutination values sharply increased by 60, 54, 38, and 0 seconds beyond the initial ones, or an average increase of 220 per cent. While it was improbable that this large increase might have been due to the difference in agglutinin production of the 2 groups of eggs, this could only be demonstrated by actual tests. This was done. Samples of egg water from the same freshly shed eggs were tested with freshly shed sperm, all dilutions being the same. The agglutination values were only little more than the initial tests, namely 20, 28, 26, 20 seconds respectively. In other words, the difference in agglutinability between the two fresh sperms was only 5, 12, 4, and 4 seconds, or 36 per cent. These differences are attributable primarily to differences in agglutinin production in the two batches of eggs. The difference in agglutinability of the same sperm when fresh and when 6 hours old was 220 per cent. This marked difference cannot be attributed to difference in the eggs but to the change in the sperm during 6 hours of ageing.

When the dry sperm was 24 hours old, the sperm were no longer agglutinable.[1] The sperm moderately overripe when shed, differed from the ripe sperm in reaching its maximum agglutinability precociously, *i.e.*, about the sixth hour, and in more rapidly decreasing thereafter (Figure III*b*).

SPERM OVERRIPE WHEN SHED.

In other experiments the sperm were very overripe when shed. This was determined by (1) the distinct brown color of the dry sperm instead of the light cream color of ripe sperm; (2) the less viscous condition of the dry sperm in contrast to the very viscous or "dry" condition of ripe sperm; (3) the short viability and (4) the early cessation of movement. The sperm in Experiment 23, the best in the day's collection of sea urchins, was overripe by these tests. In Experiment 22, the sperm originally good was precociously deteriorated by the high temperature that prevailed during the greater part of the experiments, namely, 29° C. In both these experiments there was no evidence of an increase in agglutination values with ageing of the sperm. In both experiments there was a slow, direct and definite lowering of agglutination values with ageing of sperm. In Experiment 23 the sperm of 2 males, namely A and B, were tested separately with the egg waters of three females. The sperm were allowed to age for 3 hours. One sperm, A, was overripe when shed, the other, B, not overripe. The overripe sperm when 3 hours old gave values equal to the initial tests, +1 per cent, the non-overripe sperm gave substantially increased agglutination values, 57 per cent. The overripe sperm was again tested when 5, 6, 8 hours old and gave progressively lower agglutinations, namely, −5, −25, −37, −100 per cent respectively. When 24 hours old no agglutination occurred. The ripe sperm, on the other hand, when 19 hours old, gave with the same egg waters very substantial increases in agglutination, namely, 46 per cent.

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[1] It should be noted that the eggs produced sufficient agglutinin, for, with ripe fresh sperm, agglutination was 28, 16, 13 and 0 seconds respectively.

In Experiment 22 the original tests gave agglutination values of 21, 26, and 18 seconds respectively. When the sperm were aged at a temperature of 29° C. for 3 hours, there was a decrease of −9, −6, and −3 seconds, or a decrease of 27 per cent. When aged 5 hours at this temperature there was no further change. At the end of 8 hours there was a decrease of 47 per cent. See Figure III*c*.

It is evident that differences in the degree of overripening of sperm, when shed, gives rise to marked differences in agglutination, just as in the case of eggs. The behavior in both eggs and sperm is dependent on (1) the degree of overripeness at the time of shedding, (2) the ageing or time after shedding, (3) the physical conditions in which they are aged, such as temperature.

Sperm, ripe when shed, and aged under conditions not too injurious shows a progressive increase in agglutination values. This increase begins about 3 hours after the beginning of the experiment, the time varying with the condition of the sperm when shed. Maximum values were reached about the 24th hour. Ninety-seven per cent. of the tests gave maximal values when sperm was 24 hours old.

On the other hand, sperm overripe when shed, or ripe sperm aged at high temperatures, gave no increase in agglutination values with ageing of the sperm. In only one instance was there a negligible increase of 2 seconds. The others decreased progressively in agglutination values with ageing of sperm.

While overripe sperm decreased continuously, ripe sperm underwent a cyclical rhythm, increasing progressively during the first 28 hours, decreasing progressively thereafter.

Agglutination of Ageing Sperm by Ageing Eggs.

The previous two sections led to the conclusion that ageing of eggs or of sperm markedly and progressively prolonged the agglutination reaction, provided the germ cells were not too overripe when shed. It might be urged that in these experiments there was the unpredictable variation due to the use of different eggs or of different sperm at successive ages.

This source of variation is eliminated when the same eggs and the same sperm are used in the same dilutions at successive ages. The data are presented in Table V.

To interpret the results it is again necessary to separate the experiments in accordance with the condition of the germ cells at the beginning of the experiment. In Group A the eggs and the sperm were both in good physiologic condition. In Groups B and C one or the other germ cell was overripe when shed. In Group D both germ cells were overripe.

GROUP *A*. EGGS AND SPERM RIPE WHEN SHED.

In Experiment 9, the egg to water ratios were the same throughout and hence no corrections need be made. In this experiment the freshly shed germ cells gave 17, 13, and 22 seconds for the three females tested. When the same egg water (now 23 hours old), in the same dilution as in the initial test, was used with the same sperm (now 26 hours old), the agglutination increased by 42, 6, and 44 seconds respectively, or 177 per cent. This increase is due to the changes in the ageing sperm. Further support of this conclusion is given by the experiment in which the same 24 hour old sperm was tested by freshly shed eggs of 3 females. The results were very similar, namely, 184 per cent. greater than the initial test. When equally aged eggs were tested with freshly shed sperm the values were only 50 per cent. greater than the initial tests. This gives the measure of change in ageing eggs. When, however, the same 24 hour eggs were tested with the same 24 hour sperm the values were not only much greater than the initial ones, much greater than when the eggs alone were aged, but as great or greater than when sperm alone were aged, namely 192 per cent.[1] It seems that the large increase in agglutination was determined largely by the ageing of sperm rather than by the eggs, and that aged eggs tested by aged sperm gave higher agglutination values than when eggs or sperm were aged.

In Experiment 10 the freshly shed eggs of four females were tested separately by a suspension of a 4 hour dry sperm. The

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[1] The agglutination of female No. 2 is given as 9 seconds. This is probably an error and should read 19 or 29 seconds with corresponding greater average per cent. increase.

TABLE V.

SHOWING CHANGE IN AGGLUTINATION TIME WHEN SAME EGGS AND SPERM ARE TESTED AT SUCCESSIVE AGES.

Exp. No.	Age of		Aggl. Time in Sec.				Egg Water Ratio.	Egg Water Dilution.	Corrected Values.				Average Change in %.
	Eggs.	Sperm.	♀ 1	♀ 2	♀ 3	♀ 4			♀ 1	♀ 2	♀ 3	♀ 4	
							Group A. Eggs and Sperm Ripe when Shed.						
9a	1 hr.	1 hr.	17	13	22		1 : 3, 3, 3	1/80					
j	24	26	59	19	66		1 : 3, 3, 3	1/80					+ 177
d	24	24	82	9[2]	61		1 : 3, 3, 3	1/80					+ 192
g	24	1	24	23	31		1 : 3, 3, 3	1/80					+ 50
b	1	24	58	20	70		1 : 3, 3, 3	1/80					+ 184
100	1	4	12	13	11	15	1 : 6, 6, 6, 4	1/320					
q	22	25	31	20	22	26	1 : 6, 6, 6, 4	1/320					+ 94
t	22	1	21	13	17	25	1 : 6, 6, 6, 4	1/320					+ 49
r	1	25	15	16	14	17	1 : 3, 3, 5, 8	1/320	11	12	13	21	+ 11
w	22	25 + 1[1]	18	13	17	20	1 : 6, 6, 6, 4	1/320					+ 33
19*a*	1	1	29	21			1 : 2, 2	1/320					
c	26	26	50	39			1 : 2, 3	1/320		42			+ 84
b	1	26	50	30			1 : 3, 3	1/320	55	33			+ 76
f	26	1	18	20			1 : 2, 3	1/320		22			− 20
24*a*	1	1	17	29	28		1 : 3, 3, 3	1/320					
h	3	3	15	26	20		1 : 4, 5, 5	1/320	17	35	28		+ 8
i	5	5	18	23	27		1 : 6, 6, 7	1/320	27	34	46		+ 44
	1	5	25	32	30		1 : 6, 6, 7	1/320	36	48	51		+ 82
	5	1	17	30	27		1 : 6, 6, 7	1/320	25	45	46		+ 56

TABLE V.—(*Continued*).

Exp. No.	Age of		Aggl. Time in Sec.				Egg Water Ratio.	Egg Water Dilution.	Corrected Values.				Average Change in %.
	Eggs.	Sperm.	♀ 1	♀ 2	♀ 3	♀ 4			♀ 1	♀ 2	♀ 3	♀ 4	
Group B. Overripe Sperm and Ripe Eggs.													
25*a*	1	0	15	16	22	16	1 : 3, 3, 4, 3	1/300					
l	3	3	13	18	16	11	1 : 3, 3, 4, 3	1/300					− 16
m	6	6	66	40	38	36	1 : 3, 3, 2, 3	1/300			29		+ 147
o	24	24	0	6	14	24	1 : 6, 5, 4, 7	1/300		8	14	30	− 36
k	1	24	0	0	0	0	1 : 3, 3, 4, 3	1/300					− 100
g	24	1	0	14	15	16	1 : 6, 5, 4, 7	1/300		17		32	− 7
*z*1	24	3		28			1 : 5	1/300					
*z*2	24	20		55			1 : 5	1/300					
23*a*	1	0	19	18	19		1 : 2, 2, 3	1/300					
r	3	3	33	93	34		1 : 2, 2, 3	1/300					+ 185
s	5	5	15	29	27		1 : 4, 3, 3	1/300	22	31	27		+ 43
u	8	8	13	15	12		1 : 7, 5, 6	1/300	26	29	18		+ 30
w	24	24	0	0	0		1 : 6, 6, 6	1/300					− 100
*w*1	24	20	8	11	0		1 : 6, 6, 6	1/300	16	22			+ 2
*w*5	1	24	0	0	0								− 100
*w*4	1	20	0	0	0								− 100
*w*3	1	18	30	24	14								+ 21
	24	1	10	18	10		1 : 6, 6, 6	1/300	20	36	15		+ 48

TABLE V.—(*Continued*).

Exp. No.	Age of		Aggl. Time in Sec.				Egg Water Ratio.	Egg Water Dilution.	Corrected Values.				Average Change in %.
	Eggs.	Sperm.	♀ 1	♀ 2	♀ 3	♀ 4			♀ 1	♀ 2	♀ 3	♀ 4	
							Group C. Overripe Eggs and Ripe Sperm.						
5*a*	1	1	17	22	13	26	1 : 4, 3, 4, 1	1/80					
b	4	4	13	13	7	12	1 : 8, 8, 8, 4	1/80	19	20	11	24	− 5
c	6	6	26	12	10	31	1 : 4, 4, 4, 2½	1/80	26	13	10	46	+ 17
d	22	22	21	9	8	12	1 : 5, 5, 10, 4	1/80	23	12	17	24	− 2
f	1	22	25	18	11	60	1 : 4, 3, 4, 1	1/80					+ 46
h	22	1	17	13	14	0	1 : 5, 5, 10, 4	1/80	19	18	28		− 16
20*p*	1	1	23	23			1 : 5, 3	1/320					
q	4	4	23	17			1 : 5, 4	1/320		19			− 6
r	6½	6½	26	21			1 : 6, 4	1/320	27	23			0
s	9	9	14	14			1 : 5, 4	1/320		16			− 40
t	23	23	26	24			1 : 5, 4	1/320		27			+ 6
o	1	23	37	39			1 : 5, 4	1/320		42			+ 58
n	1	9	29	25			1 : 5, 4	1/320		28			+ 14
j	23	0	20	14			1 : 5, 4	1/320		16			− 28
							Group D. Overripe Eggs and Overripe Sperm.						
3*a*	1	1	8	7	16		1 : 5, 5, 2	1/120					
b	3	3	8	8	21		1 : 5, 5, 2	1/120					+ 31
c	6	6	11	8	15			1/120					+ 9
g	24	24	0	0	0		1 : 5, 6, 4	1/120					
j	24	1		7	9		1 : 5, 6, 4	1/120		8	13		− 9
f	1	24	0	0	0		1 : 5, 6, 4	1/120					− 100
22*a*	1	1	21	26	18		1 : 3, 3, 3	1/320					
b	3	3	24	29	27		1 : 3, 3, 3	1/320					+ 23 [3]
l	5	5	11	12	14		1 : 8, 5, 5	1/320	19	15	17		− 21 [3]
m	8	8	10	11	9		1 : 10, 6, 5	1/320	20	17	13		− 22 [3]
g	8 [3]	1	17	18	13		1 : 10, 6, 5	1/320	34	27	20		+ 24
j	1	8 [3]	13	12	9			1/320					− 47

[1] Mixture of 25 hr. and 1 hr. sperm.

[2] Probably should read 19 or 29.

[3] Temp. 29° C.

agglutination values were 12, 13, 11, and 15 seconds respectively. The second test was made 21 hours later with the same germ cells. These 22 hour eggs, as in other experiments, were washed just as in the initial test, the supernatant liquid was in the same egg to water ratio for the same time (1 hour), samples of the egg water were diluted to the same concentration, and immediately tested. Suspensions of the dry sperm, now 25 hours old, were freshly made in the same 1 per cent. concentration, with the same stock of "sterile" sea water. Under these comparable conditions the agglutination values were larger in every instance, viz., 19, 7, 11, and 11 seconds respectively, *i.e.*, an average increase of 94 per cent. Other samples of the same egg waters (of these 22 hour eggs) were then tested with freshly shed sperm (Exp. t) and gave longer agglutination values than the initial tests by 49 per cent. This is in accordance with the results already described for ageing eggs. When the 25 hour sperm (Exp. r) was tested with freshly shed eggs the values were only 11 per cent. greater than the initial tests. This is interpreted to mean that the sperm had passed the peak of agglutinability. When, however, these same overripe eggs and sperm were tested together the values were far greater than when the eggs or the sperm were aged alone, namely 94 per cent. greater than the initial tests.

In Experiment 19 the freshly shed germ cells gave a 29 and a 21 second agglutination reaction. When the same eggs, 26 hours old, were tested by freshly shed sperm, the values were 20 per cent. *below* the initial ones, indicating low agglutinin production of the senescent eggs. The sperm were equally aged (26 hours old), yet when tested with freshly shed eggs the values were 76 per cent. above the initial ones. When this overripe sperm was used with the overripe eggs the values were *somewhat larger* than when aged eggs or aged sperm alone were used, for the increase was now 84 per cent. greater than the initial tests.

In Experiment 24 the germ cells were tested when 1, 3, and 5 hours old, only. When both germ cells were 3 hours old there was little change, +8 per cent. When 5 hours old the increase was greater, namely 44 per cent.

It is concluded that old eggs tested with old sperm gave in every instance values greater than either old eggs or old sperm and much greater than the initial values. The results are plotted in Fig. VII.

OVERRIPE SPERM AND RIPE EGGS. GROUP *B*.

In Experiment 12 the sperm were overripe when shed. When the germ cells were freshly shed the agglutination values were 12, 13, 11, and 15 seconds respectively. When 25 hours old no agglutination occurred in any of the 4 females. The 25 hour sperm was then tested with freshly removed eggs of 4 females. No agglutination occurred in any test, showing that the sperm were excessively overripe and not agglutinable. When, however, the 25 hour eggs, in the same egg water ratio, were tested with freshly removed sperm, the agglutination values were 12, 18, 15, and 7 seconds. This showed that the eggs were still liberating abundant agglutinin, and therefore not responsible for the lack of agglutination in the previous tests with old sperm. The old sperm had deteriorated precociously, due to the aged condition at the beginning of the experiment.

In Experiment 25 the sperm were only moderately overripe when shed. Tests were made at 1, 3, 6, and 24 hours. The 3 hour germ cells gave somewhat lower values than the initial ones, viz., −16 per cent. When 6 hours old there was a marked increase of 147 per cent. When the eggs and the sperm were 24 hours old, the agglutination values dropped sharply to a level below the initial ones, namely −36 per cent. Part of this sharp drop is due to the fact that the eggs of female No. 1 were senescent and liberated no agglutinin. The other 3 females liberated nearly as much agglutinin as in the initial test. The old sperm were also quite senescent. For, when tested with the freshly shed eggs of 4 females, they did not agglutinate in a single instance. Yet when these senescent sperm were used with old but not senescent eggs, they agglutinated 0, 8, 14, and 30 seconds respectively.

The precocious senescence of the sperm was due to the overripe condition at the time of shedding and to subsequent ageing. Such senescent sperm attain maximal agglutination values early, not when 24 hours old, as in experiments with ripe germ cells, but when 6 hours old.

It should also be noted that when the 24 hour eggs were tested with 1 hour sperm the agglutination lasted 17 seconds. When 3 hour sperm was used the agglutination lasted 28 seconds. When 20 hour sperm was used the agglutination lasted 55 seconds. When, however, 24 hour sperm was used no agglutination occurred. Agglutination had increased with ageing of sperm, until the maximum was reached, about 20 hours after shedding. Then followed a very rapid decrease, so that 4 hours later no agglutination occurred.

In Experiment 23 the sperm were overripe when shed. The results are in substantial agreement with the previous experiment. Maximal values occurred precociously, *i.e.*, when the germ cells were only 3 hours old. The increase averaged 185 per cent. When 5 hours old the agglutination values were only 43 per cent. above initial ones, when 8 hours old, 30 per cent. above, and, when 24 hours old no agglutination occurred in any of the tests. Further tests showed (Exp. W^3, W^4, W^5) that the sperm had suddenly become non-agglutinable between the 18th and the 20th hour. The eggs were in moderately good physiologic condition when 24 hours old, giving an average of 48 per cent. above initial values, but the sperm were quite senescent even when 20 hours old. Yet these senescent sperm were not only agglutinated by these old eggs but agglutinated for almost as long periods as in the initial tests.

The results are plotted in Fig. 7*b*.

Some change has taken place in addition to the increase in agglutinin of ageing eggs. For the increase in agglutination is greater than can be accounted for by the agglutinin alone. The additional factor or group of factors not only lengthens the agglutinating time but causes sperm to agglutinate after they have ceased to do so with freshly shed eggs. This factor occurs concomitantly with ageing of sperm.

Overripe Eggs and Ripe Sperm. Group *C*.

When the eggs were overripe but the sperm not overripe at the time of shedding, the resulting behavior is different.

In Experiment 5 the eggs of the 4 females were overripe when shed. The sperm were ripe. These germ cells gave initial values of 17, 22, 13, and 26 seconds respectively. When 4 hours old the values were practically the same, *i.e.*, −5 per cent. When 6 hours old there was a small increase, *i.e.*, 17 per cent. When 22 hours old the values had returned to the initial ones, −2 per cent. When the germ cells were tested separately, the old eggs with freshly shed sperm averaged 16 per cent. below the initial values. This low average was in part due to the entire lack of agglutinin production by female No. 4, and in part due to the low agglutinin production of the other senescent eggs. On the other hand the 22 hour old sperm tested by freshly shed eggs gave 46 per cent. higher values than in the initial tests. This showed that the sperm were not senescent. The lack of agglutination in the previous 22 hour test was clearly due to insufficient agglutinin and hence the real agglutinability of the sperm could not be evidenced.

The eggs in Experiment 20 were also in poor condition when shed. These eggs gave at the end of 4 hours essentially the same as the initial values, *i.e.*, −6 per cent. When 6½ hours old there was practically no change, 0 per cent. When 9 hours old there was a decrease of −40 per cent. But when 23 hours old there was a return to initial values, *i.e.*, +6 per cent. The explanation is found in testing the germ cells separately. The 9 hour sperm tested with freshly shed eggs gave an increase of 14 per cent. The 23 hour sperm gave an increase of 58 per cent. over initial values. On the other hand the 23 hour eggs, tested by freshly shed sperm, gave a decrease of −18 per cent. The low values for these old eggs indicate the degree of their senescence. The high values for the old sperm indicate corresponding lack of senescence. When such senescent eggs are tested by old but non-senescent sperm, the values are not increased as was the case when old but non-senescent eggs were used. In other words *sufficient agglutinin is necessary for the sperm to manifest the marked and progressive increase in agglutinability with overripening.*

It appears that while agglutinin of eggs decreased with ageing, the agglutinability of the sperm was markedly increased. The resultant values are the summation of the amount of agglutinin given off by the eggs and of the agglutinability of the sperm at the time of the experiment.

When eggs and sperm were both ripe when shed, and then tested at successive ages, agglutination increased at first because agglutinin of eggs increased. Later the increase in agglutination was due to the late increase in agglutinability of sperm. Maximal increases are high and late. When the eggs were overripe and the sperm ripe at the time of shedding, maximal agglutination was low and early, due to progressively lowered agglutinin production. When sperm were overripe and eggs ripe at the time of shedding, maximal agglutination occurred early, but the values were high. This is due to adequate agglutinin but lowered agglutinability of the sperm.

Overripe Eggs and Overripe Sperm. Group *D*.

When eggs and sperm were both overripe when shed (Exp. 3), or, when the germ cells were ripe but precociously aged by high temperature (29° C.), Experiment 22, there was with ageing a smaller and earlier increase in agglutination.

In Experiment 3, when the germ cells were 3 hours old, the values were 31 per cent. above the initial tests; when 6 hours old, only 9 per cent. above initial values; and when 24 hours, no agglutination occurred. The rapid decrease is apparently due to early senescence of the sperm. The 24 hour sperm were clearly senescent for they did not agglutinate with freshly shed eggs. Similarly, in Experiment 22, agglutination increased +23 per cent. when the germ cells were 3 hours old, decreased to −21 per cent. when 5 hours old, and to −23 per cent. when 8 hours old.

The explanation is due, as before, to precocious reduction of agglutinability of the sperm and to precocious reduction in agglutinin production of eggs.

Reference to Figs. 1 to 7 will bring out sharply the differences in behavior due to the degree of overripeness of each of the germ cells. The ordinates represent change in agglutination time, in per cent. as compared with the initial tests. The abscissas represent age of germ cells in hours.

In other words, in order that the increasing phase in the agglutination phenomenon be manifested, it is necessary that both the germ cells be not too overripe at the time of shedding.

The evidence demonstrates the cyclical change in agglutinin production by ageing eggs, (2) the cyclical change in agglutinability of ageing sperm. (3) When both eggs and sperm aged,

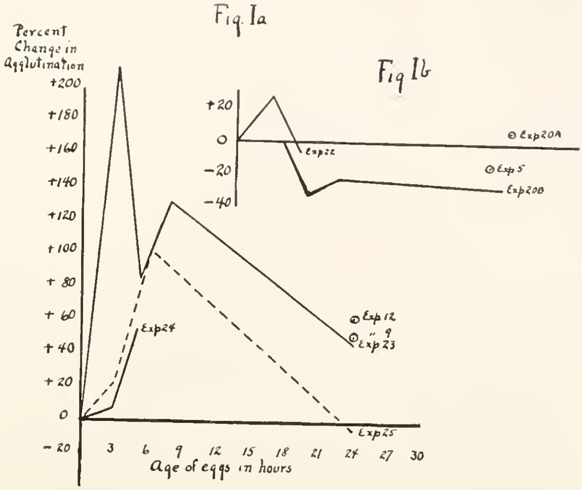


FIG. 1*a*. The agglutination cycle when eggs alone were aged, and when such eggs were in good physiologic condition at the time of shedding. Each graph is the average for all females of a series. These graphs show the marked and progressive increase and subsequent decline in values.

FIG. 1*b*. The agglutination cycle for ageing eggs when the eggs were overripe at the time of shedding. There is a small or no initial increase, and a slow decrease in values thereafter.

agglutination is a resultant of the amount of agglutinin produced by the eggs at a given age, and the degree of agglutinability of the sperm at that age.

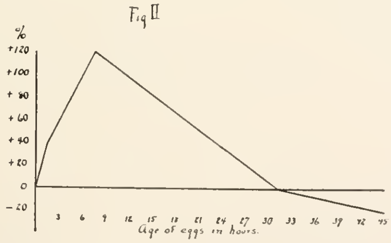
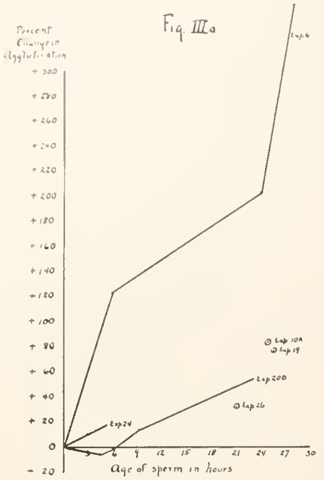


FIG. 2. The probable average agglutination cycle of ageing eggs from maturation to death, showing the marked progressive and early increase in agglutinin production.



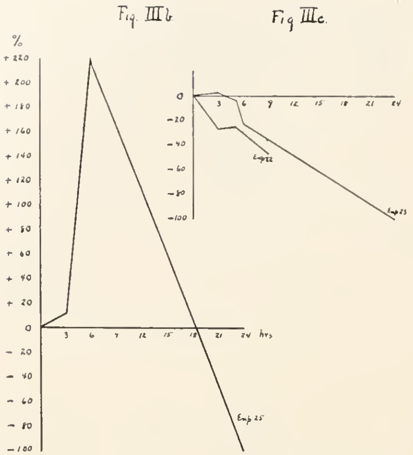


FIG. 3*a*. The agglutination cycle when sperm alone are aged and when the sperm are ripe at the time of shedding. This figure shows the marked, progressive, but slower increase in agglutinability with age.

FIG. 3*b*. The curve for ageing sperm, when the sperm is moderately overripe when shed, showing the precocious and large increase in agglutinability, and the very rapid decrease thereafter.

FIG. 3*c*. The agglutination cycle for ageing sperm, when the sperm were more overripe at the time of shedding. This shows a progressive decrease only.

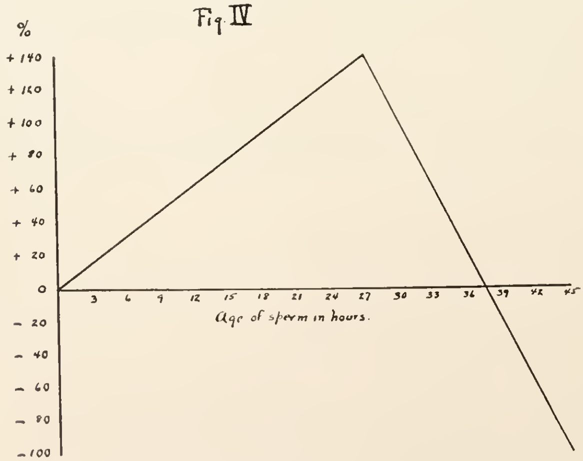
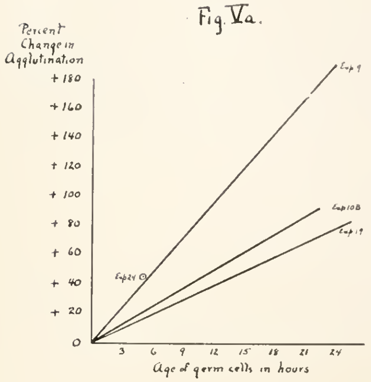


FIG. 4. The probable average agglutination cycle for the agglutinability of the sperm from maturation to death. The sperm cycle attains its maximum more slowly and decreases thereafter more quickly than the egg cycle.



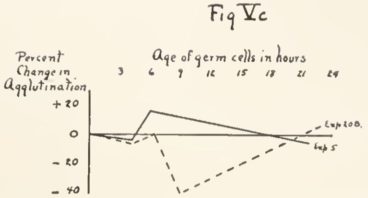
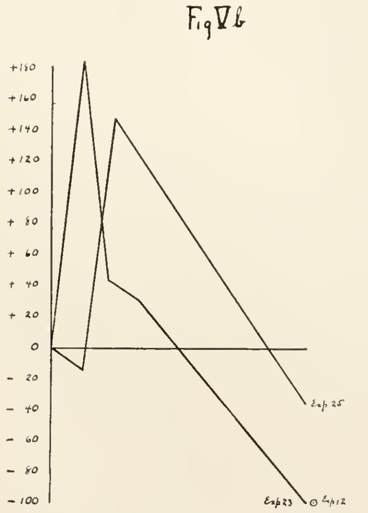


FIG. 5*a*. The agglutination changes when both the germ cells are ripe at the time of shedding and the same germ cells used at successive ages. The graphs strongly suggest the large influence of the sperm. Note the marked increase in agglutination.

FIG. 5*c*. When eggs were overripe at the time of shedding no marked nor progressive increase occurred, due to insufficient agglutinin.



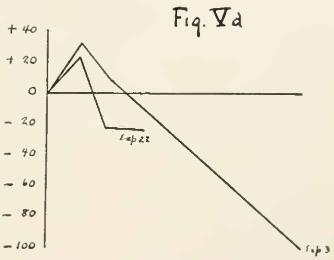


FIG. 5*b*. When the sperm were overripe at the time of shedding the maximum is reached earlier, 3 to 6 hours instead of 24–28 hours; the decline thereafter is correspondingly more rapid.

FIG. 5*d*. When the sperm and eggs were overripe at the time of shedding there is a small and early increase, due to insufficient agglutinin and low ability of sperm to react.

DISCUSSION.

To understand the behavior of the germ cells one must remember that they are matured before shedding, that the interval between maturation and shedding may be brief or may be very long, that storms and other factors extend this period (Goldforb, '29). Within the body overripening progressively takes place, the degree of overripening being a function of time since maturation, temperature and H-ion concentration of the body fluid or surrounding sea water. I wish to stress the fact that, in addition to a genetic variation in the germ cells from different individuals, there is a very much larger variation at the time of shedding, due to the extent of overripeness. This large variation in physiologic condition occurs even in freshly collected specimens from which the germ cells were freshly shed and immediately tested. One finds all degrees of change from underripe to extremely overripe germ cells. This large variability in "normal" germ cells occurs at the height of the breeding season (Goldforb, '29). Whether "ovary" or "shed" eggs are used seems not to be important. The degree of overripeness is however of the utmost importance.

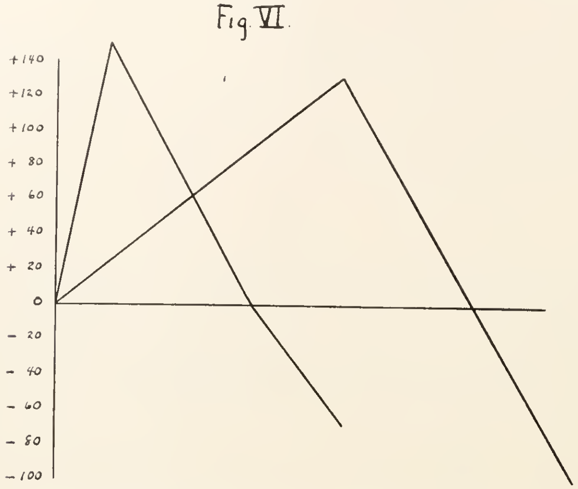


FIG. 6. Gives schematically the trend, when both germ cells were ripe (left) and when partially overripe (right) when shed.

These differences in physiologic age give rise to corresponding differences in behavior before and after fertilization and during development. It is therefore necessary in comparing germ cells from different individuals to choose by suitable tests those which are in nearly the same stage of ripeness or overripeness.

Fig VII.

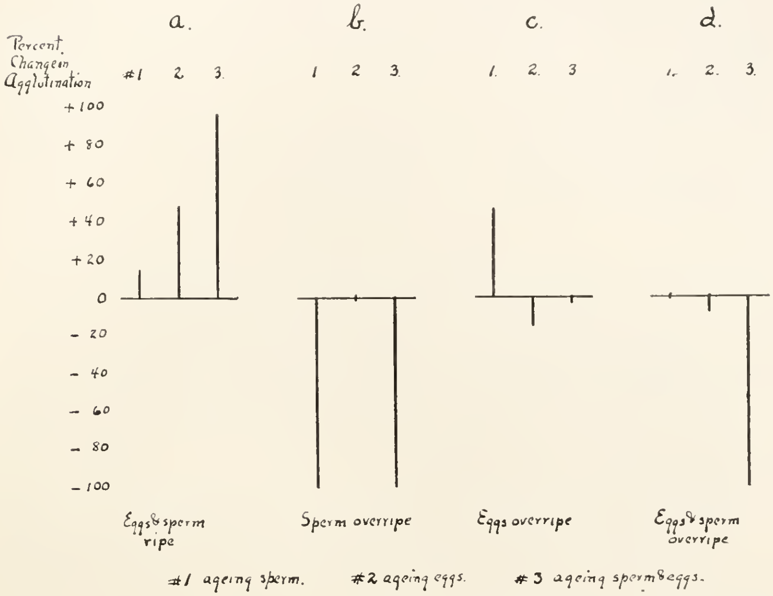


FIG. 7*a*. A typical experiment in which the sperm alone, No. 1, eggs alone, No. 2, and both, No. 3, were aged 24 hours. In the last instance the agglutination is much greater than either of the other two. Both germ cells were ripe when shed.

FIG. 7*b*. A typical experiment in which the sperm alone were overripe when shed. When 24 hours old this sperm did not agglutinate with either fresh eggs, No. 1, or with old eggs, No. 3. The old eggs produced almost as much agglutinin as in the initial test, No. 2. The total lack of agglutination was not due to absence of agglutinin but to inability of sperm to agglutinate.

FIG. 7*c*. The eggs were overripe when shed. The old sperm agglutinate well, No. 1, the old eggs produce little agglutinin, No. 2. This prevents the old sperm from manifesting its increased agglutinability, No. 3.

FIG. 7*d*. Sperm and eggs were both senescent when shed. Total lack of agglutination is due to low agglutinin and to low agglutinability of the sperm.

The changes which occur with overripening are the same within the body and outside the body. These changes may be summarized as follows:

A. 1. Size of eggs increases with overripeness.

2. Color of eggs fade.

3. Shape of eggs becomes less globular, more ellipsoid.

4. Viscosity decreases (Heilbrunn, '15, '26).

5. Jelly layer decreases.

6. Fusion of eggs increases (Morgan, '95, '24, Driesch, '00, Goldforb, '13, deHaan, '13, Goldforb, '18).

7. Fertilization membrane forms closer to egg, is more scalloped and is not found in extremely overripe eggs (Loeb, '03, Harvey, '10, '14, Lillie, '14, Heilbrunn, '15, Goldforb, '17, '18*a*, '18*b*, Lillie and Just, '24).

8. Artificial parthenogenesis increases (Matthews, '01, Lillie, R. S., '14, Loeb, '03).

9. Self fertilization increases (Morgan, '05, '10, '24, Fuchs, '14, '15).

10. Cross fertilization increases (Hertwig, O., and R., '86, '87, Vernon, '00, Tennant, '10, Kupelweiser, '09, '12).

11. Polyspermy increases (Hertwig, '85, Lillie, F. R., '19).

12. Segmentation increasingly irregular (Hertwig, '85).

13. Developmental energy decreases (Vernon, '99, Lillie, '14, Goldforb, '18, Newman, '21).

14. Change in larvæ (Tennent, '10, '11, Koehler, '15).

15. Dry sperm less creamy, more tan, less viscous.

16. Dry sperm loses mobility and viability.

17. Sperm suspensions decrease in mobility and viability (Gemmill, '00, Lillie, '14).

18. Sperm suspensions decrease in metabolism (Cohn, '18).

B. 1. Increasing or more rapid fertilizability, then decreasing or slower fertilizability (Gemmill, '00, Morgan, '04, '05, '10, Cohn, '18, Goldforb, '18*a*, '18*b*, Paspaleff, '27).

2. Parthenogenesis increases, then decreases (Lillie, R. S., '08, '15, Herlant, '18, '19, Loeb, '03).

3. Rate of fertilization membrane formation increases, then decreases (Morgan, '04, '05, '10).

4. Rate of segmentation increases, then decreases (Fuchs, '14, Goldforb, '18*a*, '18*b*).

5. Cleavage irregular then regular (Gemmill, '00).

6. Egg improves, then deteriorates (Lillie, R. S., '08, '15).

7. Agglutinin formation by eggs increases, then decreases (Goldforb, '29, Hinrichs, '27).

8. Agglutinability of sperm increases, then decreases with further overripening.

The *A* group of changes represents the phase stressed by previous workers, namely a progressive change in one direction with overripening.

The *B* group represents a cyclical change, including a prior or vitalizing phase, followed by a decreasing or senescent phase. The symptoms of physiologic change in Group *B* strongly indicate that germ cells are not at their optimum, whether for fertilization, agglutination or segmentation, when they have just matured. A certain degree of ageing or overripening is necessary for optimal results. Beyond this optimum there is a return to the initial condition and then senescence.

An ever increasing number of factors have been shown to be cyclical. The agglutination cycle is but one of many such cyclical changes in ageing germ cells. Its significance lies in part in the fact that quantitative values are more readily obtained in this than in many other characteristics.

Beginning with maturation there occurs a progressive improvement or increase until a maximum is attained. After this there is a progressive senescence or decrease in agglutinin production by the eggs and in agglutinability of the sperm.

This increasing phase in the life of the germ cells is too much ignored or not appreciated. The maximum reactivity of the egg, in respect to agglutinin production, fertilizability, segmentation, parthenogenesis occurs not when first matured but when partially overripe. This optimal stage may be synchronous with shedding or may occur many hours earlier or later. The determining factor is not the time after shedding, but the time after maturation, the OH ion concentration of the sea water and the temperature. If eggs are retained within the body for long periods, then only the decreasing phase is manifested.

The sperm undergoes a similar cyclical change. It has generally been assumed that agglutination time is determined by the amount of agglutinin, that the sperm is a constant, reacting in accordance with the dose of agglutinin.[1]

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[1] Lillie, '14, does record that an increase of sperm was required to produce agglutination when the sperm were aged.

I have shown that "normal" sperm from different individuals are not in a similar condition even when freshly removed from freshly collected males at the height of the breeding season. They undergo, within the body of the male, all degrees of overripeness. Depending on the degree of overripeness will depend the duration of the agglutination reaction, when the other conditions are comparable.

When the sperm are recently matured, and tested at successive intervals, there is clear evidence of a progressive, marked yet slow increase in agglutinability followed by a rapid decrease. These changes in agglutinability occur even when the same egg waters are used, without eggs and without visible jelly. They may be due either to a substance which activates the egg to greater agglutinin production, or which activates agglutinin to greater activity, or a substance which makes the sperm more sensitive to a given dose of agglutinin.

That the substance is not secreted entirely by the eggs is shown by the large increase in agglutination when the same egg water was used at successive ages, and in which there were no eggs nor jelly. The possibility that dissolved jelly, containing agglutinin, may be in the egg water from old eggs, is practically eliminated. For at each age the eggs were carefully washed, and the subsequent procedure was the same.

It is improbable that agglutinin is activated or increased by the hypothetic substance. For aged sperm, which are no longer agglutinated by ripe eggs (with plenty of agglutinin), can be made to agglutinate by overripe eggs with little agglutinin.

The facts seem to point to a substance secreted by ageing sperm or a physiologic change in ageing sperm that makes them more agglutinable to a given dose of agglutinin.

The further analysis of the cause or causes of increased agglutinability is deferred to the next study.

One can hardly escape the conclusion that agglutination is not a function of a constant sperm reacting with the same intensity to a given quantity of agglutinin, but that agglutination is a function of many variables, including (1) cyclical increase, then decrease in agglutinin production by ageing eggs, (2) cyclical increase, then decrease in agglutinability of ageing sperm, (3) different rate of change in overripening eggs and of sperm.

CONCLUSIONS.

Ageing Eggs.

1. When freshly shed eggs were allowed to overripen, there was a progressive and a very marked increase in agglutination time. The maximum was reached in 3 to 5 hours. With further overripening there was a progressive decrease until the sperm were no longer agglutinated.

2. Eggs overripe when shed, and allowed to further overripen, disclosed little or none of the increasing phase. Only the second or decreasing phase was evidenced.

3. Overripeness within the body gave rise to the same kind and degree of change in eggs as overripening outside of the body.

4. The cyclical change in agglutination is due to an increasing liberation of agglutinin. With further overripening the decrease in agglutination is due not to disintegration and liberation of anti-agglutinins but to decreasing liberation of agglutinins. With yet further overripening the anti-agglutinins come into play.

5. The behavior of eggs from different individuals can be understood only when the exact physiologic condition of the germ cells is known, when shed.

6. The cyclical behavior of eggs in respect to agglutination is paralleled by other cyclical manifestations of overripening, such as rate of fertilization, membrane formation, rate of cleavage, etc.

Ageing Sperm.

1. Agglutination is conditioned not only by the degree of overripeness of the eggs but also of the sperm.

2. Sperm from freshly collected, freshly opened *Arbacia*, freshly prepared under strictly comparable conditions, and tested at each successive age by freshly shed eggs, agglutinated for increasing periods of time. The increase in agglutination began about the third hour, reached a maximum far beyond the initial values about the 24th hour, and thereafter decreased progressively.

3. When the sperm were overripe at the time of shedding, or precociously aged by high temperature, corresponding portions of the first or increasing phase did not take place. When more

overripe at the time of shedding, only the decreasing phase occurred.

4. The degree of overripeness of sperm at the time of shedding, as well as of eggs, was determined by a series of independent tests.

5. When conditions are strictly comparable the agglutinability of sperm depended upon the degree of overripeness of the sperm when shed, and with the degree of further overripening after shedding.

Ageing Eggs Tested by Ageing Sperm.

1. When the same eggs and sperm were used at successive ages, under strictly comparable conditions, not only was there a progressive and marked increase in agglutination values, but the increase was greater than with either overripe eggs or overripe sperm alone. This occurred provided the germ cells were freshly matured at the time of shedding.

2. When the sperm alone were overripe at the time of shedding there was an earlier increase.

3. When the eggs only were overripe at the time of shedding there was little or no change in values, due to insufficient agglutinin.

4. When eggs and sperm were both overripe at the time of shedding there was only the decreasing phase, due to insufficient agglutinin and to inability of sperm to change.

5. The agglutination phenomenon is conditioned by (*a*) the degree of overripeness of eggs and of sperm at the time of shedding, (*b*) the differences in the cyclical rate of change of sperm and of eggs, (*c*) time, (*d*) conditions of ageing, such as temperature, OH ion concentration.

6. Eggs increase in agglutinin production. Sperm increase in agglutinability with ageing. The two cycles are not synchronous. The resulting agglutination depends upon the extent of change in the eggs and in the sperm. The amount of change is a summation of these factors.

7. Sperm is not a fixed or neutral agent reacting in the same way to the same dose of agglutinin. Sperm is a varying agent, either secreting increasing amounts of a substance or substances

which accelerate agglutinin formation, or sperm undergoes a physiologic change, with overripening, as a result of which they become increasingly reactive to a given dose of agglutinin.

The analysis of this substance or physiologic change will be made in the next study.

BIBLIOGRAPHY.

Buchanan, R. E. J. Bact., 1919, 4.

Clowes, G. H. A., and Smith, H. W. Am. J. Physiol., 1923, 64.

Cohn, E. J. Biol. Bull., 1918, 34.

Driesch, H. Arch. f. Ent., 1900, 10.

De Haan, B. Arch. f. Ent., 1913, 36.

Ficai, F. Pathologica, 1912, 4.

Foa, C. Arch. Ital. de Biol., 1918, 68.

Fuchs, H. M. Arch. f. Ent., 1914, 40.

Fuchs, H. M. J. Genet., 1915, 4.

Gemmill, J. F. J. Anat. and Physiol., 1900, 34.

Gies, W. J. Am. J. Physiol., 1901, 6.

Goldforb, A. J. Anat. Record, 1917, 11.

Goldforb, A. J. Proc. Nat. Acad. Sc., 1917, 3.

Goldforb, A. J. Biol. Bull., 1918, (35.)

Goldforb, A. J. Biol. Bull., 1918, (34.)

Goldforb, A. J. Biol. Bull., 1929, 57.

Gray, J. Q. J. Micr. Sc., 1915, 61,

Heilbrunn, L. V. Biol. Bull., 1915, 29.

Heilbrunn, L. V. J. Exp. Zoöl., 1926, 44.

Herlant, M. Arch. Zoöl. Exp., 1918, 57.

Herlant, M. C. R. Acad. Sci., 1919, 169.

Hertwig, O. Jena Zeit., 1885, 18.

Hertwig, O. and R. Jena Zeit., 1886, 19.

Hertwig, O. and R. Jena Zeit., 1887, 20.

Hinrichs, M. A. Biol. Bull., 1927, 53.

Just, E. E. Biol. Bull., 1919, 36.

Kabeshima, T. Zeit. f. Immunitatsf., 1913, 8.

Koehler, O. Zeit. Ind. Abst. u. Vererb., 1915, 15.

Kupelweiser, H. Arch. f. Entw. Mech., 1909, 27.

Lillie, F. R. J. Exp. Zoöl., 1914, 16.

Lillie, F. R. Biol. Bull., 1915, 28.

Lillie, F. R. Problem of Fertilization, 1919.

Lillie, F. R., and Just, E. E. General Cytology, 1924.

Lillie, R. S. J. Exp. Zoöl., 1908, 5.

Lillie, R. S. J. Exp. Zoöl., 1914, 16.

Lillie, R. S. Biol. Bull., 1915, 28.

Lillie, R. S. Am. J. Pnysiol., 1916, 40.

Loeb, J. Mechanical Conc. of Life, 1912.

Loeb, J. Artificial Parthenogenesis and Fertilization Changes, 1913.

Loeb, J. The Dynamics of Living Matter, 1906.

Matthews, A. P. Am. J. Physiol., 1901, 6.

McGregor, A. S. M. J. Path. and Bact., 1910, **14.**
Morgan, T. H. Arch. f. Ent., 1895, **2.**
Morgan, T. H. J. Exp. Zoöl., 1904, **1.**
Morgan, T. H. Biol. Bull., 1905, **8.**
Morgan, T. H. Arch. f. Ent., 1910, **30.**
Morgan, T. H. J. Exp. Zoöl., 1924, **40.**
Morgan, T. H. Sci. Monthly, 1924, **18.**
Mudd, B. H. and S. Proc. Soc. for Exp. Biol. and Med., 1929, **26.**
Newman, H. H. J. Exp. Zoöl., 1921, **33.**
Ostwald, W. Biochem. Zeit., 1907, **6.**
Paspaleff, G. Pub. Staz. Zool. Napoli, 1927, **8.**
Richards, A., and Woodward, A. E. Biol. Bull., 1915, **28.**
Robertson, T. B. J. Biol. Chem., 1912, **12.**
Robertson, T. B. Arch. f. Ent., 1912, **35.**
Sampson, M. M. Biol. Bull., 1926, **50,** 202.
Sampson, M. M. Biol. Bull., 1926, **50,** 301.
Tennent, D. H. Arch. f. Entw. Mech., 1910, **29.**
Tennent, D. H. Carnegie Inst. Washington, 1911.
Vernon, H. M. Proc. Roy. Soc. B., 1899, **65.**
Vernon, H. M. Arch. f. Entw. Mech., 1900, **9.**
Winkler, H. Nachrichten d. Ges. d. Wiss. Göttingen, 1900, **2.**

FACTORS THAT CHANGE AGGLUTINABILITY OF AGEING SPERM.

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By a previously described technique (Goldforb, '29*a*), agglutination of sperm by egg water could be measured with an average experimental error of 1 second or 4.5 per cent. With this technique freshly shed eggs from freshly collected and freshly tested sea urchins (*Arbacia punctulata*) were separately tested by freshly shed sperm, under strictly comparable conditions. These "normal" germ cells varied from 11 to 2,300 per cent. in agglutination time. This large variation was due in small part to germinal differences and in large part to wide differences in the degree of overripening of the germ cells at the time of shedding.

Later studies (Goldforb, '29*b*) showed that when eggs or sperm or both were not too overripe, at the time of shedding, there was, with ageing, a progressive and marked increase in agglutination time. The evidence compelled the conclusion that ageing eggs liberated increasing amounts of agglutinin, and that ageing sperm either secreted increasing amounts of a substance that increased the agglutination, or, that ageing sperm underwent a physiologic change that made them increasingly susceptible to a given dose of agglutinin.

The present study aims to determine which of these two possibilities actually obtains. The experiments were performed at the Marine Biological Laboratory at Woods Hole, Massachusetts, during the summers of 1924 and 1926. My thanks are due to the Directors for the facilities of the laboratory.

Experiments with Ageing Sperm Suspensions.

In preliminary experiments, samples of the same sperm suspension and the same egg water solution were tested 10 to 50 minutes after the initial test. In a considerable number of instances the later test gave increased agglutination values. Exps. 5, 12, and 25 may serve as illustrations.

TABLE I.

SHOWS AN INCREASE IN AGGLUTINATION TIME WHEN SAMPLES OF THE SAME SPERM AND EGG CULTURES WERE USED 10 AND 20 MINUTES AFTER THE INITIAL TESTS.

Exp. No.	Age of			♀ No.	Agglutination in 1/80 Egg Water.				
					Initial Test in Sec.	10 Min. Later		20 Min. Later	
	Eggs.	Sperm.	Egg Water.			Sec.	% Increase.	Sec.	% Increase.
5A	4 hrs.	4 hrs.	1 hr.	1	11	13	18	19	72
				2	13	14	7	14	7
				3	7	7	0	7	0
				4, 5, 6, 7[1]	12	16	33	21	75
B	1	22	21	1	24	26 +	8		
				2	13	23 +	76		
				3	8	26 +	225	80	900
				4, 5, 6, 7[1]	54	57	55	60	111
C	$6\frac{1}{2}$	22	$11\frac{1}{2}$	1	0	0	0		
				2	0	12	1200		
				3	0	13	1300		
				4, 5, 6, 7[1]	17	17	0		
D_1	22	22	1	1	19	19	0		
				2	9	7	− 22		
				3	9	12	33		
				4, 5, 6, 7[1]	12	0	0		
D_2	22	22 + 1[2]	1	1	37	37	0		
				2	0	12	1200		
				3	12	13	7		
				4, 5, 6, 7[1]	10	19	90		
D_3	22	1	1						
				2	13	12	− 7		
				3	17	11	− 35		
				4, 5, 6, 7[1]	0	0	0		

[1] Eggs of females 4, 5, 6 and 7 were combined.

[2] Old and fresh sperm combined.

TABLE II.

SHOWS INCREASED AGGLUTINATION VALUES WHEN SAMPLES OF THE SAME OVERRIPE SPERM SUSPENSIONS ARE USED AT SUCCESSIVE INTERVALS.

Exp. No.	Age of			♀ No.	Agglutination in Sec. in 1/320 Egg Water Solution.					
	Eggs.	Sperm.	Egg Water.		Initial Test.	10 Min. Later.	15 Min. Later.	35 Min. Later.	55 Min. Later.	% Increase.
12.1	1 hr.	25 hrs.	25 hrs.	1	0				17	1700
				2	0				13	1300
				3	0				18	1800
				4, 5, 6	0				8	800
12.3	1	25	1	1	3			11		269
				2	0			5		500
				3	0			0		0
				4, 5, 6	0			10		1000
12.2	25	25	1	1	0			9		900
				2	0			12		1200
				3	0			0		0
				4, 5, 6	0			16		1600
12.4	1	1	25	1	22			0		− 2200
				2	42			17		− 60
				3	13			9		− 30
				4, 5, 6	17			20		17
12.5	25	1	1	1	22			15		25
				2	18			22 +		22
				4, 5, 6	7			24		240

TABLE II.—(*Continued*).

Exp. No.	Age of			♀ No.	Agglutination in Sec. in 1/320 Egg Water Solution.					
	Eggs.	Sperm.	Egg Water.		Initial Test.	10 Min. Later.	15 Min. Later.	35 Min. Later.	55 Min. Later.	% Increase.
12.6	1	1	1	1	0			9		900
				2	6			0		– 600
				3	0			0		0
				4, 5, 6	17			11		– 35
25.3	0	6	1	1	0	0				0
				2	20	30				50
				3	18	23				27
				4	0	0				0
25.6	0	24	1	1	0		0			0
				2	0		28			2800
				3	0		16			1600
25.6	24	24	1	1	0		30			3000
				2	28		34			21
				3	47		40+			
25.6	0	3	1	1	0		11			1100
				2	24		28			16
				3	25		48			92
				4	12		43			258

In Experiment 5*A* (Table I) a fresh suspension of a 4 hour dry sperm was tested separately, with the egg waters of 4 females. The agglutination values were 11, 13, 7, and 12 seconds respectively. Ten minutes later samples of the same cultures gave 2, 1, 0, and 4 seconds *longer agglutinations* than the first tests, or an increase of only 16 per cent. Other samples of the same sperm suspension tested after 10 more minutes gave 8, 1, 0, and 9 seconds more than the initial tests, or an average *increase of* 42 *per cent.*

When a 22 hour sperm was used with freshly shed eggs (Exp. 5*B*) the initial agglutinations for the four females were 24, 13, 8, and 54 seconds respectively. Ten minutes later, samples of the same cultures gave increases of 2, 10, 18, and 3 seconds respectively, or 33 per cent. After 10 more minutes the increases were far greater, namely 126 per cent. When intermediate aged (6½ hours old) eggs were tested by 22 hours old sperm the first test gave 0, 0, 0, and 17 seconds, the second test 10 minutes later gave 0, 12, 13, and 17 seconds, *i.e.*, an increase of 147 per cent. When, however, freshly shed sperm was used (Exp. *D*3) no increase in agglutination occurred at the later test. When old and fresh sperm were combined (Exp. *D*2) there was again an increase in agglutination values, ten minutes later of 37 per cent.

Similar results occurred in Experiment 12 (Table II). Suspensions of a 25 hour dry sperm did not agglutinate with freshly shed eggs, but 55 minutes later agglutinated 17, 13, 18, and 8 seconds respectively (Exp. 12.1). In Experiment 12.3 the initial values were 3, 0, 0, and 0 seconds. After 35 minutes the values were 11, 5, 0, and 10 seconds respectively, an increase of 766 per cent. When 25 hour old eggs were used, the initial values were 0, 0, 0, and 0 seconds. The later values were 9, 12, 0, and 16 seconds (Exp. 12.2). Ten out of the twelve tests with the 25 hour old sperm gave material increases in agglutination.

On the other hand the freshly prepared suspensions of ripe sperm with ripe eggs, Exps. 12.4 and 12.6, gave little increased or much decreased agglutination. The average values were −13 per cent. and −61 per cent.

It appears that sperm in standard 1 per cent. suspension changed *within 10 minutes, changed further within the next 10 to 45 minutes, with corresponding increase in agglutination values. The change occurred much more markedly in overripe than in ripe sperm.* The change occurred whenever the eggs were not so overripe that not enough agglutinin was liberated to activate and agglutinate the sperm.

In Exp. 25 similar results were obtained. The eggs and sperm were in good physiologic condition when shed. The sperm was used when 3, 6, and 24 hours old. Marked increases in agglutination occurred in 10 to 15 minutes after the sperm suspension was prepared. These increases occurred in 6 out of 9 tests. The other 3 tests gave no agglutination in either the first or the second tests. In other instances, when no agglutination occurred in the initial test, the second test gave long agglutinations. The average increases were 39, 39, 113, and 1466 per cent. respectively.

Other experiments corroborated these results and led to a more detailed study of ageing suspensions of sperm.

In Experiment 11*A* (Table III) both kinds of germ cells were six hours old. Six females were used. Females Nos. 1, 2, and 3 were tested separately, 4, 5, and 6 together. The temperature was 21° C. with an increase of ½° C. during the 2½ hours of the experiment. Tests were made 15 to 30 minutes apart, with samples of the same sperm suspension and of the same egg water solution. The successive agglutination tests for female No. 1 were 13, 15, 19, 19, 26, 19, 20, 20, 15, and 16 seconds respectively. *There was an unmistakable increase in values with ageing of the sperm suspension.* Maximum values with egg water of ♀ 1, occurred not when first tested but 75 minutes later, and the increase was 100 per cent. During the subsequent 75 minutes there was a slow and progressive decrease, which did not reach the initial values at the close of the experiment, 165 minutes later.

The egg waters of females No. 2, No. 3, and Nos. 4, 5, 6 combined gave similar results. The average values for the 4 batches of eggs were 14.5, 17.0, 19.2, 20.2, 19.5, 19.7, 17.0, 15.7, 14.0, and 11.5 seconds at the successive intervals. The increases were 100 per cent. for female 1, 56 per cent. for female 2, 35 per cent.

Table III.

Increase in Agglutination When Samples of the Same Egg Water were Tested by Same Sperm Suspension at Successive Intervals. Temperature 20° C.

Exp. No.	Age of			♀ No.	Agglutination in Sec. in 1/320 Egg Water.										Maximal Increase.
	Eggs.	Sperm.	Egg Water.		0	15	30	50	75	90	105	120	150	165 Min.	
11*A*	6 hr.	6 hr.	1 hr.	1	13	15	19	19	*26*	19	20	20	15	16	100%
				2	16	19	22	21	18	*25*	22	20	19	18	56
				3	14	14	15	15	*18*	6	7	9	7	0	35
				4, 5, 6	15	20	21	26	21	*29*	19	14	15	12	93
				Aver. sec.	14.5	17.0	19.2	20.2	19.5	19.7	17.0	15.7	14.0	11.5	71
				Aver. inc. %		+ 17	+ 31	+ 39	+ 34	+ 36	+ 17	+ 8	− 3	−20	
11*B*	6	31	6	1	0	0	0								
				2	0	0	0								
				3	10	0	0								
				4, 5, 6	16	0	0								

					Agglutination in Sec. in 1/80 Egg Water. Temp. 20½° C.								Maximal Increase.
					0	5	15	25	50	80	100	125 Min.	
8*A*	5	29	1	1	21	50	40	70	*90* +	0			328%
				2	17	0	0	0	0	0			
				3	34	40	50 +	90 +	*95* +	6			179
				Aver. 1 & 3	27.5	35	45	80	92	0			
				Aver. inc. %		+ 27	+ 63	+ 191	+ 236	− 89			
				Sea-water					0	0			
8*B*	5	6	1[1]	1	24		24	29	34	39	37	*53*	120
				2	14		*17*	8 +	11	9	11		
				3	23		22	30	29	29	52	76	230
				Aver. 1 & 3	23.5		23	29.5	31.5	34	44.5	64.5	
				Aver. inc. %			− 2	+ 25	+ 34	+ 44	+ 89	+ 174	

[1] Not same egg water solution as in 8*A*.

TABLE III.—(*Continued*).

RANGE OF TEMPERATURE FROM 20½° C. TO 20° C.

Exp. No.	Age of			♀ No.	Agglutination in Sec. in 1/320 Egg Water at																	Maximal Increase.
	Eggs.	Sperm.	Egg Water.		0	10	20	30	45	55	70	100	120	140	160	180	200	240	260	280	305 Min.	
14*A*	1 hr.	24 hr.	1 hr.	1	30	30	36	48	53	45	60	70	80	62	42	36	26	3	0	0	0	166%
				2	26	26	28	31	43	40	46	42	40	30	27	24	19	6	0	0	0	77
				Aver.	23	23	32	39.5	48	42.5	53	56	60	46	34.5	30	22.5	4.5	0	0	0	
				Aver. inc. %	0	0	+39	+72	+108	+85	+130	+143	+160	+56	+50	+32	−2	−80	−100	−100	−100	
B[1]	1	1	1	1	29	28	21	19	20		18	17	15	11	12	11	12	10	10	10	10	0
				2	21	25	26	23	23		20	21	20	16	15	16	16	16	15	11	11	24
				Aver.	25	26.5	23.5	21	21.5		19.5	19 5	17.5	13.5	13.5	13.5	14	13	12.5	10.5	10.5	
				Aver. change %	0	+6	−6	−16	−14		−22	−22	−30	−46	−46	−46	−44	−48	−50	−58	−58	

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[1] Same egg water as in *A*.

for female 3, and 93 per cent. for females 4, 5, and 6. The average increase was 71 per cent. In every instance the maximal values did not take place at the initial test but 75 to 90 minutes later. When the experiment was terminated, after 165 minutes, the values were greater than the initial test in 2 batches,

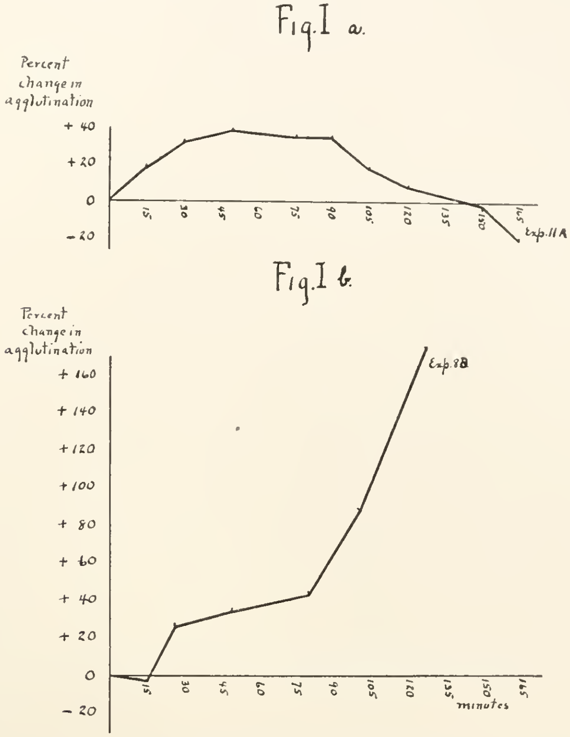

FIG. 1. Shows the slow progressive *decrease* in agglutination values when a suspension of freshly shed sperm is tested at successive intervals with samples of the same egg water.

slightly lower in 1 batch, and in 1 batch agglutination had ceased altogether (Fig. 1*a*).

In Experiment 8*B* the eggs were 5 hours old. These were tested by 2 kinds of dry sperm, one 6 hours old and the other 29 hours old. The temperature increased but ½° C. during the 2 hours of the experiment. When a suspension[1] of the 6 hour dry sperm was tested with the egg water of female No. 1, the successive values were 24, 24, 29, 34, 39, 37, and 53+ seconds, respectively. A similar increase occurred with the egg water of female No. 3, namely 23, 22, 30, 29, 29, 52, and 76 seconds. The eggs of female No. 2 were very overripe at the time of shedding, as indicated by enlarged size, oval shape, pale color, greater viscosity, rate of membrane formation, etc. The egg water of these overripe eggs gave a very small increase, then decreased in value, namely 14, 17, 8+, 11, 9, and 11 seconds.

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[1] All suspensions of sperm were 1 per cent. and tested immediately.

When eggs were not too overripe at the time of shedding, as in female No. 1 and No. 3, there was a progressive and marked increase in values with ageing of sperm suspension, namely 120 per cent. for female No. 1, 230 per cent. in female No. 3. The increase began 10 to 25 minutes after the initial test. The maximum values were not reached during the 125 minutes of the experiment (Fig. 1*b*).

Other samples of the same egg water were tested by a sperm suspension made with a 29 hour dry sperm (Exp. 8*A*). The agglutination values increased to a far greater extent than with the less overripe sperm of the previous experiment. This is in entire accord with the results obtained in ageing dry sperm (Goldforb, '29*b*). The values with the egg water of female No. 1 were 21, 30+, 40+, 70+, 90, and 0 seconds respectively. Female No. 3 gave 34, 40, 50+, 90+, 95+, and 6 seconds respectively. The increases were 328 and 179 per cent. Maximum values occurred in both, 50 minutes after the initial test. There was a very rapid decrease in values after this maximum. Female No. 2, with the very overripe eggs, gave a 17 second agglutination in the first test but no agglutination thereafter (Fig. 2).

It may be concluded from these experiments that *when eggs and sperm were not too overripe, when shed, agglutination progressively increased with ageing of the sperm suspension. The increase was more rapid and reached a greater maximum, the more overripe the dry sperm at the initial test. The change in values cannot be attributed to a change in concentration of sperm suspension, nor to a difference in agglutinin content, nor to ageing of egg water solution.* For, when such egg water was tested at successive intervals by freshly shed, freshly prepared sperm, there was no progressive increase in agglutination values. Maximal agglutination with ageing sperm occurred not at the initial test but 50 to 125 minutes later. The increase was 35 to 328 per cent.

Ageing sperm suspensions gave a similar, progressive and marked increase in agglutination values as did ageing of dry sperm (Goldforb, '29*b*). Ageing sperm suspensions gave, however, a much quicker increase.

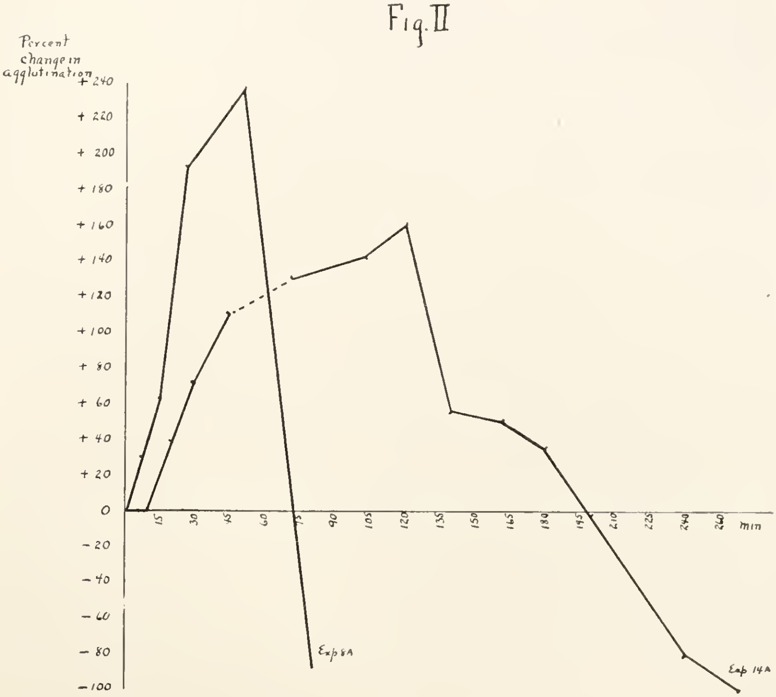

FIG. 2. Shows the slowly *increasing* agglutination values, when partially over-ripe (6 hours old) dry sperm is used.

In Experiment 14*A*, the ageing sperm suspension was tested for a longer period (305 minutes). Two kinds of sperm were used, freshly shed and 24 hour dry sperm. These were tested by the same egg water solutions from the freshly shed eggs of two females. The egg water was more diluted (1/320) than in the other experiments, which made for greater accuracy. The temperature changed but $\frac{1}{2}$° C. during the five hours of the experiment. Successive tests were made 10 to 25 minutes apart.

The results obtained with the different samples of the same ageing suspensions of overripe (24 hour) dry sperm conform in all essentials with those in previous experiments. There was a marked and progressive increase in agglutination with ageing of sperm suspension. This increase began 10 to 20 minutes after the initial tests. Maximal values were reached 120 and 70 minutes after the first tests, and were 166 and 77 per cent. greater. Thereafter the values decreased steadily. When the sperm suspension was 260 minutes old, agglutination ceased (Fig. 2).

The parallel experiment with samples of the same egg water solution but tested with non-overripe (freshly shed) sperm gave very different results. Female No. 1 gave at successive intervals *no progressive increase* as in the case of overripe sperm, but on the contrary a progressive decrease, as described by Lillie, '14, '15, Cohn, '18, Lillie and Just, '24. The values were 29, 28, 21, 19, 20, 18, 17, 15, 11, 12, 11, 12, 10, 10, 10, and 10 seconds respectively. The values for female No. 2 showed the same progressive decrease, after a brief, small increase. This small and early increase of 6 per cent. is probably of no significance. The values were 21, 25, 26, 23, 23, 20, 21, 20, 16, 15, 16, 16, 16, 15, 11, and 11 seconds respectively (Fig. 3).

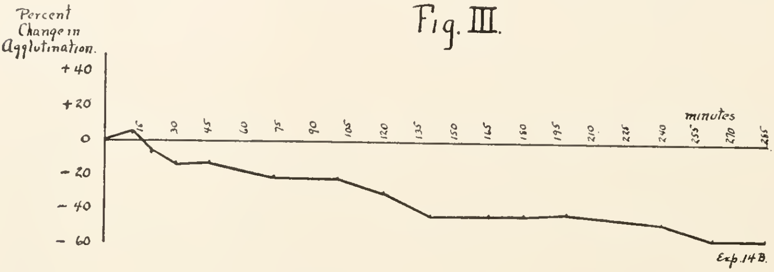

FIG. 3. These curves represent the behavior of dry sperm 24 and 29 hours old. The ageing suspension gave rise to a *more rapid and greater increase* than 6 hour dry sperm. The more rapid and greater increase in 8*A* may be due in part to the older dry sperm.

Sperm too overripe, *i.e.*, 31 hour dry sperm (Exp. 11) did not agglutinate at all.

The data are plotted in Figs. 1 to 3.

The increase in agglutination either did not occur or only slightly, when the dry sperm were not overripe at the beginning of the experiment. Nor did it occur when the sperm or the eggs were so senescent that agglutination did not occur at all, as in Exp. 11*B* and in female No. 2 of Exp. 8*A*. But agglutination did increase when the dry sperm was in intermediate stages of overripeness.

The close agreement in the results with ageing dry sperm (Goldforb, '29*b*) and with ageing sperm suspensions is most striking. The difference lies only in the rate of increase which is so much faster in ageing sperm suspensions than in ageing dry sperm.[1]

It is known that sea water dilutes the H ion concentration of the sperm culture, thereby activating the sperm. But activation by sea water neither gave rise to, nor increased, the agglutination values. Hence the increase in values with ageing of sperm suspension must be attributed to causes other than H ion concentration *per se*.

In searching for the cause or causes of this phenomenon I have excluded an increase in agglutinin as a factor. For the same egg water was used in successive tests, and there were neither eggs nor visible jelly in such solutions. I have excluded the effect ageing of the egg water solution. For when such egg water solution was tested at each successive interval by freshly prepared ripe sperm there was no progressive increase in agglutination values. Temperature was eliminated as a factor, for not only was the change but $\frac{1}{2}$° C., but the increase in agglutination occurred both when the temperature increased and when it decreased. A change in concentration of sea water was also eliminated.

The factor or factors that made for increasing agglutination must be sought in the sperm. If the sperm secretes a substance with ageing, it is not one which activates the eggs to greater agglutination liberation. For there were no eggs in the solution. Nor is there any evidence that it progressively activates the agglutinin in the solution. The other possibility is that sperm undergoes with ageing a physiologic alteration which makes the sperm increasingly susceptible to a given dose of agglutinin. The change is a cyclical one increasing with age, reaching a maximum long after the initial test, and decreasing with further ageing.

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[1] Drzewina, A., and Bohn, G. ('26), found that an increasing number of eggs (*Strongylocentrotus*) develop when dilute sperm stood for a time.

The parallelism between the known physiologic changes and ageing of sperm is most striking, such as longevity, viscosity, mobility, metabolism, permeability, agglutination. These physiologic changes are all cyclical. The agglutination increases with overripening of sperm either dry or in suspension. The dry sperm undergoes slower physiologic changes than do sperm in suspension, and shows correspondingly slower increase in agglutination values.

Is There an Increase of Sperm Substance in Increasing Concentrations of Sperm?

A number of investigators have successfully extracted a substance or group of substances from sperm. Winkler, '00, Robertson, '12, Foa, '18, Prevost and Dumas, '24, extracted a sperm substance which induced parthenogenesis. Sampson, '26, described the chemico-physical properties of a sperm filtrate which induced parthenogenesis. Dubois, '00, extracted a spermase, Ostwald, '07, a peroxidase and a catalaze. Geis, '01, and Loeb, '06, were unable to find enzyme characteristics in the sperm extract, but Richards and Woodward, '16, did. Lillie, '15, and Cohn, '18, suggested the possibility that overripe sperm may liberate a substance which aids in fertilization. Popa, '27, described a lipochromatic substance in the sperm head, which substance he believes responsible for agglutination.

Such investigations strongly suggested that overripe sperm may secrete a substance which modifies agglutination. While the evidence from ageing sperm strongly pointed to a physiologic change in the sperm, the hypothesis of increasing liberation of sperm substances with overripening was not excluded.

To find out whether the sperm liberated a substance which progressively increased agglutination, experiments were made with increasing concentrations of sperm, but with samples of the same egg water. The concentrations of sperm ranged from $\frac{1}{2}$ per cent. to 25 per cent. More than 25 per cent. could not be used for the suspensions were then too opaque to distinguish agglutinated clusters in the thick creamy mass of sperm. If the increase in agglutination in previous experiments was due to an increasing liberation of a sperm substance which energized agglutinin, one should expect that increasing concentrations of sperm should correspondingly lengthen agglutination time. If the increase is due to a physiologic change in the sperm, then an increase in concentration, *per se*, should not increase agglutination.

In Experiment 4*A* (Table IV) ripe sperm was used in ½, 1, 2, and 4 per cent. suspensions. The agglutination values in a 1/20 dilution of egg water were 19, 27, 25, 28 seconds respectively. In a 1/60 egg water dilution, and therefore more accurate, with sperm in ½, 1, 2, 4, 10, and 25 per cent. concentrations, the agglutination values were 7, 14, 13, 13, 13, and 13 seconds respectively. In a still more dilute egg water dilution, namely 1/120, and with sperm in 1, 2, 4, and 10 per cent. concentrations, the values were 9, 9, 10, and 9 seconds respectively. The egg water of female No. 2 in a dilution of 1/120 gave 17, 27, 22+, 27, 28, and 28 seconds.

Less than 1 per cent. concentration of sperm did not give full agglutination values as Lillie has already made clear. At least 1 per cent. suspension is needed. But increasing the concentration of sperm *did not* further increase the agglutination values even in the dense 25 per cent. concentration.

There was the possibility that not enough time had been allowed for the liberation of the hypothetical substance or substances from the sperm. Previous experiments, however, showed that ageing 1 per cent. sperm suspension began to increase in agglutinability within 5 to 25 minutes, and reached maximal values in 15 to 125 minutes. Ageing dry sperm showed the initial increase within 3 hours and gave maximal values in 63 per cent. of the tests when 3 to 4 hours old.

In Experiment 4*B*, therefore, the same dry sperm was used as in the previous experiment, but was now 3½ hours old. Furthermore the sperm suspension was 65 minutes old when used. This was then deemed ample time to liberate the hypothetic sperm substance. With increasing concentration of sperm there should be increasing quantities of the sperm substance, manifested in increasing agglutination values. In a 1/20 dilution of egg water and with 1, 2, 4 per cent. sperm concentrations the values were 28, 27, and 29 seconds respectively. In the more delicate tests

Table IV.

Showing No Increase in Agglutination with Increasing Concentration of Sperm. Agglutination in Seconds.

Exp. No.	Age of			♀ No.	Concentration of Sperm in %.						Egg Water Dilution.
	Eggs.	Sperm.	Egg Water.		½	1	2	4	10	25	
4*A*	1½ hr.	1½ hr.	1 hr.	1	19	27	25	28			1/20
				1	7	14	13	13	13	13	1/60
				1		9	9	10	9		1/120
				2	17	27	22+	27	28	28	1/120
4*B*	3½	3½	1	1		28	27	29			1/20 Suspension 1 hr. old.
				1		10	9	11	11		1/120 " " " "
19*A*	1	1[1]	1	1*a*	28	26	27	26	27	28	1/320 Aliquot parts
				1*b*	27	29	29	28	27	28	1/320 of eggs.
19*B*	1	1[1]	26	1	17	19	18	18	19	18	1/320
				2	11	20	19	20	21	21	1/320
19*C*	26	1[1]	1	1	17	18	18	17	18	20	1/320
				2	19	20	15+	16+	16+	18	1/320

[1] Sperm overripe when shed.

with a 1/120 egg water dilution, and with sperm concentrations of 1, 2, 4, and 10 per cent., the values were 10, 9, 11, and 11 seconds. There was clearly no evidence of a sperm substance, even after the lapse of so much time, in any of the concentrations of sperm used.

Experiment 19 is an example of another type of experiment. The sperm, when shed, were by various tests shown to be overripe (Goldforb, '29*a*, '29*b*). This overripe sperm was then tested by (*a*) egg water from freshly shed ripe eggs, (*b*) other freshly shed ripe eggs whose egg water was 26 hours old, (*c*) fresh egg water from overripe (26 hour old) eggs. If a substance is liberated by overripe sperm, it should manifest its presence in one or more of these three tests with overripe sperm. The egg water dilution was 1/320 throughout. The temperature was 22½° C. The sperm concentrations were ½, 1, 2, 4, 10, and 25 per cent., freshly prepared for each test.

In Experiment 19*A* the freshly shed ripe eggs were divided into two equal portions. The egg water of each was tested separately with freshly prepared sperm suspensions. This served to check the accuracy of the experimental method. One portion gave, in increasing concentrations of sperm, 28, 26, 27, 26, 27, and 28 seconds, the other registered 27, 29, 29, 28, 27, and 28 seconds. There is a remarkably close agreement in the two samples of eggs. There was clearly no evidence of a sperm substance which increased the duration of the agglutination phenomenon.

Comparison of Experiments 19*A*, 4*A*, and 4*B* with those experiments in which ageing sperm were used, brings out in sharpest relief the complete absence of any increase in agglutination in either ripe or in overripe sperm by merely increasing the concentration of the sperm, while progressive and marked increases occurred in 1 per cent. suspensions as they became increasingly overripe.

In Experiment 19*B* old egg water (26 hours old) was used, with the same overripe sperm, in the same concentrations. The egg water of female No. 1 gave 17, 19, 18, 18, 19, and 18 seconds respectively. Female No. 2 gave an 11 second reaction in ½ per cent. sperm suspension, and 20, 19, 20, 21 and 21, seconds in the other concentrations. The heavy concentrations of sperm cultures did not manifest any increase in agglutination in these old egg water solutions.

In Experiment 19*C* overripe (26 hour) eggs were used. Female No. 1 gave, in the increasing sperm concentrations, 17, 18, 18, 17, 18, and 20 seconds. Female No. 2 gave 19, 20, 15+, 16+, 16+, and 18 seconds. There was no increase in values when overripe eggs were used.

In Experiment 19 with ripe or overripe eggs, with fresh and with old egg water solutions, with ripe or overripe sperm, *there is no evidence of a substance liberated by sperm.*

Discussion.

There can be no doubt that agglutination was not increased with increasing concentration of sperm. On the other hand agglutination was markedly and progressively increased by ageing of sperm, either concentrated or in suspension. When increased agglutination took place, it was *not* due to a substance activating the eggs to greater agglutinin production. For the increase in agglutination values occurred in samples of the same egg water from which eggs and jelly were excluded.

It is conceivable that the substance may be modified so as to intensify the activity of the agglutinin in the egg water. This was suggested as a possibility by F. Lillie, '19. In the first place there is no known basis for this hypothesis. Much more pertinent is the fact that one should expect on this hypothesis an increase in agglutination values in those experiments in which increasing concentrations of sperm were used. But no such increase occurred.

It should be recalled that students of the agglutination phenomenon in bacteria have come to a similar conclusion, namely, that a physiologic change or changes in the bacteria are responsible for the change in agglutination (McGregor, '10, Ficai, '12, Kabeshima, '13, Buchanan, '19).

The observation that loss of fertilization occurs more quickly than loss of motility (Lillie, F., '14, '15, '19, Lillie, F., and Just, E., '24) is paralleled by the observed fact that loss of agglutination occurs more quickly than loss of motility. It is conceivable and probable that loss of fertilization and of agglutination are associated with the physiologic changes described above.

Sufficient time elapsed to permit the substance, if present, to be liberated into the culture medium. Ripe eggs liberate a substance, agglutinin, in about 15 minutes. Sperm on account of their small size and relatively large surface should liberate their substance more quickly. Yet in 3½ hours, ripe dry sperm did not give any evidence of a substance that increases agglutination, nor did the overripe sperm in Experiments 19*A*, 19*B*, 19*C*.

Nor may one assume that the hypothetic activating substance is formed and liberated increasingly with ageing sperm. For even in overripe sperm, Experiment 4*B*, the increasing concentrations give no evidence of increase in agglutination.

It is known that sperm give off CO_2, and the carbonic acid thus formed modifies the relative OH ion concentration of the medium. The work of Loeb, '03, '14, Lillie, '14, '15, Cohn, 18, Lillie and Just, '24, clearly indicates that the carbonic acid' plays an important rôle in *decreasing the activity and thereby increasing the longevity of the sperm.* It is known that *carbonic acid induces aggregation but there is no evidence of carbonic acid increasing agglutination.*

It is therefore concluded that carbonic acid is not responsible for the increasing agglutination values.

It is furthermore concluded that there is no definite evidence of a substance liberated by sperm which increases the intensity or the duration of agglutination.

The facts strongly suggest a cyclical physiologic change or changes in the sperm, which make the sperm more reactive to a given dose of agglutinin.

It is known that both eggs and sperm undergo cyclical changes. This is evidenced by changes in metabolism, in gelation of surface, in viscosity, in permeability, etc., all of which are associated with overripening. Overripening eggs show progressive solution of jelly, liberation of agglutinin, or increased rate of agglutinin production. Hence overripening eggs give rise to increased agglutination values. Overripening sperm, undergoing similar physiologic cyclical changes, increases in agglutinability.

When therefore ageing eggs are tested by ageing sperm the agglutination values are greater than when either eggs or sperm alone are aged. This is true not only for ageing dry sperm but for ageing sperm suspensions.

It is this physiologic change in sperm as well as in eggs that gives rise to the initial improving stage, evidenced here in increasing agglutination. This physiologic change, continuing, leads to an optimum condition of the germ cells and then to senescence.

The cyclical physiologic change in eggs is manifested by the cyclical increase then decrease in agglutinin liberation. The cyclical physiologic change in sperm is manifested by increase then decrease in agglutinability.

The life cycle of the sperm may be abbreviated, *i.e.*, the physiologic changes may be hastened by dilution, by heat, by excess OH ions, etc. With such increase there is a corresponding precocious increase in agglutinability. Freshly matured dry sperm may not show evidences of a change for 3 hours. Overripe dry sperm show evidences of a physiologic change at once. Suspensions of ripe sperm show little evidence of a physiologic change during the whole life of the sperm.[1] Moderately overripe sperm in the same dilution show evidences of a physiologic change in 15 to 25 minutes (Exp. 8*B*, 11*A*). Sperm more overripe show evidence of a physiologic change sooner, namely, 5 to 20 minutes (Exp. 8*A*, 14*A*).

If the factor which gives rise to increased agglutinability be a sperm secretion, there should occur with increasing concentration of sperm correspondingly increased agglutination. This does not take place. On the other hand, if the factor be a physiologic change in the sperm, then increasing concentration of freshly prepared sperm should produce no change in agglutination, which is exactly what takes place.

I am therefore compelled to conclude that the increase in agglutination so marked in ageing sperm, whether dry or in suspension, is due to a physiologic change in the sperm, which change makes them more susceptible to a given dose of agglutinin.

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[1] It is improbable that the change may occur within the first 10 minutes.

The discovery of agglutinins secreted by the eggs led to the assumption that the egg plays the dominant rôle in agglutination.[1] Lillie, '17, dealing with other phases of germ cell behavior, states that "the old idea that sperm supplies organs or substances necessary for activation must be abandoned. The egg possesses all substances needed for activation. The sperm is an inciting cause of these reactions within the egg system. . . ." It has been assumed that secretions of varying amounts of agglutinin were the determining factor in changing agglutination. The sperm merely reacted to varying quantities of agglutinin.

My studies have shown that the egg does not decrease, but on the contrary *increases the rate of agglutinin liberation* with age until an optimum is reached several hours after maturation. These studies also showed that *sperm are not constant* as heretofore assumed, but that sperm is equally variable, increasing in agglutinability with overripening, until an optimum is reached 6 to 24 hours after maturation (dry sperm) or 70 to 120 minutes after preparation of the suspension. This cyclical change in agglutinability appears not to be due to a secretion, but to a physiologic change which makes sperm increasingly agglutinable by a given dose of agglutinin.

Summary.

Previous studies demonstrated that with increasing overripening of eggs or of sperm, or both, agglutination values increased correspondingly.

The present study demonstrates that precocious overripening of sperm, by dilution, gave rise to a correspondingly precocious and markedly progressive increase in agglutination.

This precocious increase occurred when the dry sperm were overripe. The increase began in 5 to 20 minutes after the initial test. Maximum values occurred 15 to 125 minutes after the initial test. The increase in values ranged from 77 to 328 per cent. The greater the overripeness of the dry sperm the greater the increase, the earlier the maximum and the sooner the cycle ended.

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[1] For full bibliography and review I refer to Lillie, Problems of Fertilization, '19, Lillie and Just in General Cytology, '24, and to Morgan, Experimental Embryology, '27.

Suspensions made of ripe sperm either did not increase at all or only slightly. The agglutination values then decreased progressively. This is the phase heretofore described.

The cyclical increase and decrease in agglutination was not due to a change in the eggs, nor jelly, nor temperature, nor to a changed OH ion concentration.

This increase in agglutination is not due to a substance liberated by sperm. For agglutination values were not increased when the concentration of sperm was increased from 1 to 25 per cent., the maximum concentration usable. The values were the same whether ripe or overripe sperm were used.

Sufficient time elapsed for the substance, if present, to be liberated.

The CO_2 liberated by sperm has considerable effect upon the activity of the sperm, upon aggregation, but does not increase agglutination.

The cyclical agglutination change is due to a physiologic change in ageing sperm, manifested in a changing metabolism, gelation, viscosity, permeability, and in an increased reaction of sperm to a given dose of agglutinin.

This cyclical change is paralleled by the eggs, which increases agglutinin liberation.

It is therefore concluded that the cyclical physiologic change in overripening is responsible for the improving phase in both germ cells, and with subsequent senescence. This physiologic change is analogous to that in agglutinating bacteria.

Sperm are not a biologic constant, as heretofore believed, but undergo marked physiologic changes with corresponding marked and progressive increase in agglutinability followed by progressively decreasing agglutinability.

BIBLIOGRAPHY.

Buchanan, R. E. J. Bact., 1919, **4**.
Cohn, E. J. Biol. Bull., 1918, **34**.
Drzewina, A., and Bohn, G. Compt. Rend. Soc. Biol., 1926, **95**.
Dubois. Compt. r. des. Sc. de la Soc. de Biol., 1900, **52**.
Ficai, G. Pathologica, 1912, **4**.
Foa, C. Arch. Ital. de Biol., 1918, **68**.
Gies, W. J. Am. J. Physiol., 1901, **6**.
Goldforb, A. J. Biol. Bull., 1929*a*, **57**, 333.
Goldforb, A. J. Biol. Bull., 1929*b*, **57**, 350.

Kabeshima, T. Zeit. f. Immun. Forsch., 1913, **8.**
Lillie, F. R. J. Exp. Zoöl., 1914, **16.**
Lillie, F. R. Biol. Bull., 1915, **28.**
Lillie, F. R., and Just, E. E. General Cytology, 1924.
Lillie, F. R. Problems of Fertilization, Chicago Press, 1917.
Loeb, J. Arch. Ges. Physiol., 1903, **99.**
Loeb, J. The Dynamics of Living Matter, N. Y., 1906.
Loeb, J. Arch. f. Entw., 1914, **38.**
Loeb, J. Amer. Naturalist, 1915, **49.**
McGregor, A. S. M. J. Path. and Bact., 1910, **14.**
Morgan, T. H. Experimental Embryology, Columbia Univ. Press, 1927.
Ostwald, W. Biochem. Zeit., 1907, **6.**
Popa, G. T. Biol. Bull., 1927, **52,** 223.
Popa, G. T. Biol. Bull., 1927, **52,** 238.
Prevost and Dumas. Annales des Sc. Naturelles, 1924, **2.**
Richards, A., and Woodward, A. A. Biol. Bull., 1916, **30.**
Robertson, T. B. J. Biol. Chem., 1912, **12.**
Robertson, T. B. Arch. f. Entw., 1912, **35.**
Sampson, M. M. Biol. Bull., 1926, **50,** 202.
Sampson, M. M. Biol. Bull., 1926, **50,** 301.
Winkler, H. Nachrichten d. Ges. d. Wiss., Göttingen, 1900, **2.**

A NEW CASE OF INTERSEXUALITY IN *RANA CANTABRIGENSIS.*[1]

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The specimen to be described is an adult sexually mature wood-frog, *Rana cantabrigensis*, killed on October 26, 1928. It measures 3.7 cm. from the tip of the snout to the ischial symphysis, and is definitely and typically male in all the somatic features which are characteristic of this sex in this species. Nuptial pads are distinct and pigmented, very similar to those found in normal male frogs during this season, and the Wolffian ducts possess well-defined seminal vesicles in the usual position. The gonads appear as normal testes, which, upon a general macroscopical examination, show nothing atypical, nor any external evidence of germinal intersexuality.

Upon sectioning, both gonads are shown to be intersexual. The bulk of either gonad is composed of spermatic tissue, the histological structure of which corresponds exactly with that found in normal testes of this season. Most of the seminal tubules are thickly lined with dense bundles of spermatozoa. Examination of numerous male sex cells does not show any deviation from the normal processes of development.

The ovarian elements consist of individual ova or oöcytes, which may be designated as testis-ova (Fig. 1). The left gonad contains only one testis-ovum, deeply imbedded in the spermatic substance, while in the right gonad there is about a dozen of such cells scattered throughout. All of the ova are actually situated within the seminal tubules and lie among the male germ cells. None of them is associated with pigmentation, nor with any tissue hypertrophy, both of which are characteristic features of certain degenerative changes of germinal elements. The ova average about 200 micra in diameter, the larger ones measuring 250 micra or more. In shape the ova differ considerably, being mostly round or oval. In many places, they become much distorted in form, owing to the pressure exerted on them by the tubules in which they are contained. Occasionally they occupy the whole lumen of the tubule, causing some derangement of the male germ cells nearby. Structurally, the testis-ova are devoid of true ovarian follicles; they appear invested by a delicate non-cellular fibrous membrane. Their nuclei are distinct, with sharply defined nuclear membranes, though thin and often irregular. There is no visible evidence of any intranuclear network. The nucleus stains only slightly, while the numerous nucleoli of various sizes are intensely basophilic, aggregating mostly along the periphery. Minute granules are found imbedded everywhere in the nucleus. The oöplasm stains grayish

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[1] Contribution from the Zoölogical Laboratory of the University of Michigan.

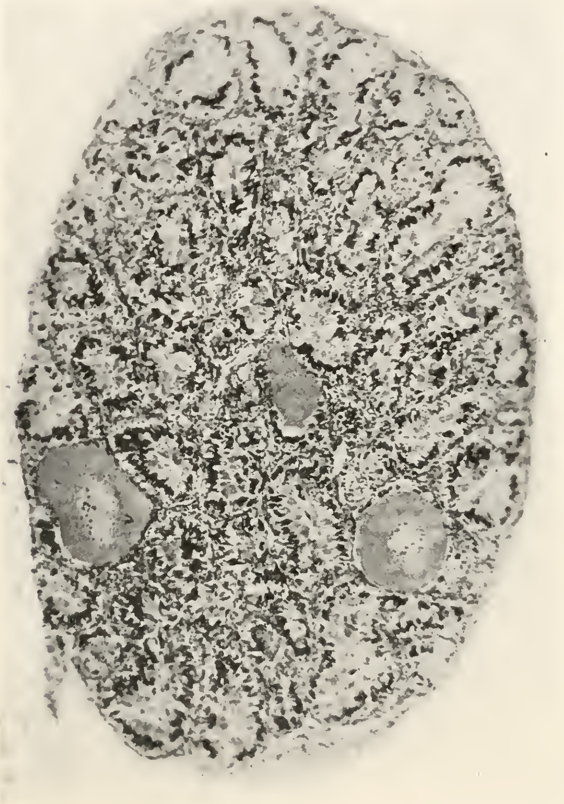

FIG. 1. A cross section of the right abnormal testis, showing three ova within the seminal tubules. Magnification approximately × 64.

and shows no vitellogenetic activity. All structures and conditions indicate a remarkable resemblance of these testis-ova to the normal young oöcytes of similar size.

From the above description, it appears that the testis-ova are of no functional value. Should they mature, there is no means of exit to the exterior, for there is no identifiable trace of Müllerian ducts. On the other hand, the presence of well-developed nuptial pads and of a perfect male accessory sex apparatus warrants the conclusion that this abnormal frog must have functioned as a male, despite the duplex condition of its gonads.

Review of Literature.

Instances of ova-containing testes have been encountered in various species of *Rana*.[1] Balbiani wrote (1879, p. 219), "Il arrive assez souvent, lorsqu'on pratique des corpes de testicules de Grenouille ou de Crapaud, même parvenus à l'âge de reproduction, de trouver dans les tubes ou les ampoules séminifères des ovules anormalement developpés, constitués identiquement comme les jeunes ovules transparents de l'ovaire de la femelle." Pflüger (1882, p. 33) found pronounced "Graaf'schen Follikel" in the testes of three-year-old Utrecht frogs. According to Spengel (1884, p. 270), Fritz Meyer had observed in the Leipzig frogs, "Hoden mit Ovarialeinschlüssen und umgekehrt Ovarien mit Hodeneinschlüssen." Marshall (1884) reported several abnormal frogs (*Rana temporaria*), one of which possessed ova-containing testes as well as Müllerian ducts. Another specimen of similar nature in the same species was found by Latter (1890). In *Rana viridis*, Friedmann (1898 *a*) described well-developed as well as degenerating ova in otherwise normal adult testes. He also observed (1898 *b*, p. 874) in the same species, "in mehreren Hoden intratubulär gelegene Gebilde, die zumindesten eine grosse Aehnlichkeit mit jungen Eizellen aufweisen." Mitrophanow (1894) noticed one true ovum and many doubtful ones in the testes of a frog (*Rana esculenta*). From references given by him and Cole (1896), Eismond (1892) had a young male frog showing testis-ova in various stages of development. In *Rana*

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[1] Some of the cases cited below may be examples of Pflüger's hermaphroditism, which is found to be of normal occurrence in certain races of frogs.

pipiens, King (1910, p. 166) reported rudimentary ova in an adult testis and Swingle (1917) large oöcytes in well-developed testes of a young "pseudo-hermaphrodite." Champy (1913, p. 103) frequently observed oviform cells in testes of *Rana temporaria* and *Rana esculenta*. Witschi (1914, p. 69; 1921, pp. 326, 328) presented cases of testis-ovum in immature *Rana temporaria*. Takahashi (1919, p. 310) found, in an otherwise normal adult male of *Rana limnocharis*, three egg-cells in the right gonad. Van Oordt (1923) discovered about 100 ova of 100–160 micra in diameter in the right testis of an adult frog (*Rana fusca*). In *Rana catesbiana*, Swingle (1926, pp. 473–474, 491, 492; Fig. 97) mentioned the occurrence of maturation cells (which are probably ovarian) in larval testes, and of oöcyte-like cells in larval and adult testes. In *Rana sylvatica*, Witschi (1929, p. 267) found young oöcytes in the early development of male gonals. Cheng (1930) described a number of intersexual gonads in tadpoles of *Rana cantabrigensis*, which exhibited varying amounts of female substance amid male tissues.

Marshall (1884), Heymons (1917), Crew (1921 *a*), and Lloyd (1929) reported cases, in which one or both of the testes, otherwise normal, were found to contain pigmented materials. These pigmented bodies are often interpreted as degenerating ova or remains of ovarian tissue. Chidester (1926) recorded two interesting specimens of *Rana catesbiana*, in which ova were found in other parts of the body, instead of in the testes, which were normal in these cases.

There is always some difficulty in making a clear-cut distinction between an ovo-testis (or ovotestis) and an ovum- or ova-containing testis. The writer has regarded an ovotestis as composed of recognizable portions of ovary and testis, while an ovum- or ova-containing testis is mainly testicular in structure, containing an ovum, ova or groups of ova in its spermatic substance. Intermediates between the two types have been encountered and can be arranged in a genetical series. There are cases on record in which one gonad is an ova-containing testis, while the other one is a more or less distinct ovotestis (Kent, 1885;[1] Cole, 1896; Crew, 1921 *a;* Woronzowa, 1926, and others).[2]

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[1] See Crew's re-description of Kent's specimen (Crew, 1921 *a*).

[2] Recently Christensen (1929) and Witschi (1929*b*) have reported new cases of intersexuality in *Rana pipiens* and in *Rana temporaria* respectively.

In toads, testis-ova have been recorded by Balbiano (1879), Hoffmann (1886), Knappe (1886), Cole (1896),[1] Ikeda (1896), Friedmann (1898 *a*), Cerruti (1907), Champy (1913), Takahashi (1915, 1919), Caroli (1925, 1926, 1927), Beccari (1925), Cunningham and Becton (1926), and Stohler (1928). In *Hyla*, Sweet (1908) found, in an adult male, imperfectly developed Müllerian ducts and 15–20 ova scattered irregularly through the substance of the testes, generally singly but sometimes in groups of two.

Discussion.

Popoff (1909) observes in the testicular tubules what he designates as "ovules mâles," each one of which eventually gives rise to "une spermatogemme et ensuite à un faisceau spermatique définitif." Swingle has argued that oöcyte-like cells are not necessarily female sex cells, especially when occurring in an otherwise male individual. Levy (1920) and Orlowski (1925) claim that the so-called eggs in the testicular tissue are nothing but "polyploide Riesenzellen." Champy (1913) presents some evidence to show that the primitive gonia in the male gonad may sometimes become hypertrophied to form oviform cells which may develop further and "aboutit à la formation d'ovocytes incontestables qu'on ne peut distinquer de ceux d'un jeune ovaire." Crew and Fell (1922) are of opinion that the ovum-like bodies in their material are mere liquefaction products of the spermatozoa.

In our specimen, the testis-ova obviously cannot be homologized with the "ovules mâles" of Popoff. They do not represent any stage in the normal development of male sex cells and there is no trace of anything resembling them in sections of normal testes of our wood-frogs. The size and morphology of the testis-ova show distinctly that they cannot be "polyploide Riesenzellen," nor coalesced masses of degenerating spermatozoa. Finally, there is no indication in the histology of the ovum-containing testes nor in the cytogenesis of the sex cells contained therein, that the testis-ova have been derived by an abnormal hypertrophy of the spermatic substance. No intermediate or transitional forms exist between the testis-ova and the male sex elements. From these considerations we may reasonably assume that the testis-ova in our frog are true ovarian cells, though immature and apparently degenerating.

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[1] Cole stated that Dr. Beard had informed him that well-developed ova occurred apparently normally in the testis of the toad quite apart from the Bidder's organ.

The occurrence of female germ cells in the testes described in this paper definitely demonstrates that male and female sex elements can, under certain circumstances, become developed and fostered in one and the same gonad of the frog. It affords additional evidence of a germinal bipotentiality, owing to which the indifferent germ cells, in the gonad of one sex, may change their course of development and assume the characteristics of the other sex. Humphrey (1929 *a*) recently reports that this tendency of reversal is a common feature in the larval testes of *Ambystoma*. The question, however, remains unsolved whether such a tendency has resulted from certain changes produced in the genital glands by environmental conditions, or from inherent peculiarities in the sex-differentiating agency. As pointed out in our previous papers (Cheng, 1929, 1930), certain abnormalities in the development of sex may probably be induced by genetical defects in the zygotic sex constitution, or in the embryonic mechanism of sex differentiation. Further evidence for this will be presented in a separate publication. Swingle (1917) is inclined to believe that an unequal distribution of chromosomes at the spermatocyte division may account for the appearance of hermaphroditic forms. Lloyd (1929) has pointed out that if the natural occurrence of hermaphrodite frogs is due to environmental influences, "it would be reasonable to expect, in any given frog population in which a single hermaphrodite occurs, a good percentage of frogs similarly affected, as presumably they would all have been subjected to the same influences. That this is not the case is shown by the rarity of hermaphrodites amongst frogs." The work of Goldschmidt and others on the gipsy moth has clearly shown that a great variety of intersexual conditions may result from genetical causes. Data from sex-intergrade pigs (Baker, 1925, 1929) lend support to the inheritance of a tendency to sexual abnormality. In Amphibians, intersexuality and sex reversal apparently can be produced artificially by various experimental means, such as parabiosis

(Burns, 1925; Witschi, 1927), gonad grafting (Burns, 1928; Humphrey, 1929 *b*), high temperature (Witschi, 1914, 1929*a*), nutritional disturbance (Champy, 1921) and the like. Crew (1921 *b*) and Witschi (1923) have performed breeding experiments on hermaphroditic frogs in an attempt to determine their genetical nature. The results obtained appear to indicate that those hermaphrodities are of the female type, but, as elsewhere pointed out by the author, do not show that they are genetically normal females, a significant fact which has been overlooked by all previous investigators. Until more is known about the genesis and genetics of sporadic intersexuality, it would seem premature to discuss whether the above described frog is genetically intersexual, or genetically male with an initial deficiency of sex differentiating substance, or genetically female transforming into a male.

The writer wishes to express his sincere gratitude to Prof. Peter Okkelberg under whom this research has been conducted.

LITERATURE CITED.

Baker, J. R.

'25 On Sex-Intergrade Pigs: Their Anatomy, Genetics and Developmental Physiology. Brit. Journ. Exper. Biol., **2**: 247–264.

'29 A New Type of Mammalian Intersexuality. Brit. Journ. Exper. Biol., **6**: 56–63.

Balbiani, G.

'79 Leçon sur la génération des Vertébrès.

Beccari, N.

'25 Ovogenesi larvale, organo del Bidder e differenziamento dei sessi nel *Bufo viridis*. (Studii sulla prima origine delle cellule genitali nei vertebrati, IV). Arch. Ital. Anat. e Embriol., **22**: 483–549.

Burns, B. K., Jr.

'25 The Sex of Parabiotic Twins in Amphibia. Journ. Exper. Zoöl., **42**: 31–89.

'28 The Transplantation of Larval Gonads in Urodele Amphibians. Anat. Rec., **39**: 177–192.

Caroli, A.

'25 Variazioni e anomalie dell'organo di Bidder, quale contributo all'erfro, ditismo della gonade degli Anfibi Anuri. Atti. R. Accad. Fisiocritici-Siena, ser. 9, **17**: 555–581.

'26 Lo studio dell'organo di Bidder in rapporto al tipo di variazione nel *B. vulgaris* Laur. e *B. viridis* Laur. Atti. R. Accad. Fisiocritici, Siena ser. 10, **1**: 331–347.

'27 Recherches sur l'organe de Bidder des Bufonidés en rapport à l'étude de l'hermaphroditisme de la gonade des amphibies anoures. Arch. Ital. Biol., **78**: 29–34.

Cerruti, A.

'07 Sopra due casi di anomalia dell'apparato riproduttore nel *Bufo vulgaris* Laur. Anat. Anz., **30**: 53–64.

Champy, C.

'13 Recherches sur la spermatogénèse des Batraciens et les éléments accessoires du testicule. Arch. Zool. Expér. et Gén., **52**: 13–304.

'21 Changement expérimental du sexe chez le *Triton alpestris* Laur. Compt. Rend. Acad. Sci., **172**: 1204–1207.

Cheng, T. H.

'29 Intersexuality in *Rana cantabrigensis*. Journ. Morph. and Physiol., **48**: 345–370.

'30 Intersexuality in Tadpoles of *Rana cantabrigensis*. Papers Mich. Acad. Sci., Arts and Letters, **11**: 353–368 (*in press*).

Chidester, F. E.

'26 Incipient Hermaphroditism in the Bullfrog. Anat. Rec., **34**: 142 (*Abstract*).

Christensen, K.

'29 Hermaphroditism in *Rana pipiens*. Anat. Rec. 43: 345–358.

Cole, F. J.

'96 On a Case of Hermaphroditism in *Rana temporaria*. Anat. Anz., **11**: 104–112.

Crew, F. A. E.

'21*a* A Description of Certain Abnormalities of the Reproductive System Found in Frogs, and a Suggestion as to their Possible Significance Proc. Roy. Phys. Soc., Edinburgh, **20**: 241–258.

'21*b* Sex-Reversal in Frogs and Toads. A review of the recorded cases of abnormality of the reproductive system and an account of a breeding experiment. Journ. Gen., **11**: 141–181.

Crew, F. A. E., and Fell, H. B.

'22 The Nature of Certain Ovum-like Bodies Found in the Seminiferous Tubules. Quart. Journ. Microsc. Sci., **66**: 557–578.

Cunningham, B., and Becton, C.

'26 The Occurrence of an Ovo-Testis in *Bufo terrestris*. Journ. Elisha Mitchell Sci. Soc., **42**: 14–15.

Eismond, M.

'92 Coupe d'un ovaire, pas completement développé, de la grenouille (avec les signes extérieurs mâles). Trav. Labor. Zoot. de Varsovie, **7**: 8 (*Original in Russian*).

Friedmann, F.

'98*a* Ueber rudimentäre Eier in den Hoden von *Rana viridis*. Arch. f. mikr. Anat., **52**: 248–262.

'98*b* Beiträge zur Kenntniss der Anatomie und Physiologie der männlichen Geschlechtsorgane. Arch. f. mikr. Anat., **52**: 856–891.

Heymons, R.

'17 Ueber hermaphroditische Bildungen bei einem Männchen von *Rana temporaria* L. Sitzgb. Gesellsch. naturf. Freunde Berlin, Jahrg. 1917, 354–368.

Hoffmann, C. K.

'86 Zur Entwicklungsgeschichte der Urogenitalorgane bei den Anamnia. Zeitschr. f. wiss. Zoöl., **44**: 570–643.

Humphrey, R. R.

'29*a* Studies on Sex Reversal in *Amblystoma.* I. Bisexuality and Sex Reversal in Larval Males Uninfluenced by Ovarian Hormones. Anat. Rec., **42:** 119–156.

'29*b* Studies on Sex Reversal in *Amblystoma.* II. Sex Differentiation and Modification Following Orthotopic Implantation of a Gonadic Preprimordium. Journ. Exper. Zoöl., **53:** 171–220.

Ikeda, S.

'96 Hermaphroditism in Toads. Zoöl. Mag. (Organ of Zoöl. Soc., Tokyo), **8:** 10–22. (*Original in Japanese.*)

Kent, A. F. S.

'85 A Case of Abnormal Development of the Reproductive Organs in the Frog. Journ. Anat. and Physiol., **19:** 347–350.

King, H. D.

'10 Some Anomalies in the Genital Organs of *Bufo lentiginosus* and Their Probable Significance. Am. Journ. Anat., **10:** 159–176.

Knappe, E.

'86 Das Biddersche Organ. Morph. Jahrb., **11:** 489–552.

Latter, O. H.

'90 Abnormal Reproductive Organs in *Rana temporaria.* Journ. Anat. and Physiol., **24:** 369–372.

Levy, F.

'20 Ueber die sogenannten Ureier im Froschhoden. Biol. Zentralbl., **40:** 29–37.

Lloyd, J. H.

'29 Hermaphroditism in the Common Frog (*Rana temporaria*). Am. Nat., **63:** 130–138.

Marshall, A. M.

'84 On Certain Abnormal Conditions of the Reproductive Organs in the Frogs. Journ. Anat. and Physiol., **18:** 121–138.

Mitrophanow, P.

'94 Un cas d'hermaphrodisme chez la grenouille. Bibliog. Anat., **11:** 32–36.

Oordt, G. J. van.

'23 Demonstratie van ein volwassen testis van *Rana fusca,* waarin een groot aantal eieren geworden wordt. Nederl. Tijdschrift. v. Geneeschunde, **66:** 2223.

'23 Das Vorhommen von Eiern in den Samenkanälchen erwachsener Amphibien. Anat. Anz., **57:** 38–44.

Orlowski, M.

'25 Sur l'origine de pseudooeufs dans le testicule de la grenouille. Kosmos, Czasopismo polskiego Towarzystwa Przyrodnikow im. Kopernika, **50:** 167–172. (*Original in Polish.*)

Pflüger, E.

'82 Ueber die das Geschlecht bestimmenden Ursachen und die Geschlechtsverhältnisse der Frösche. Arch. f. gesam. Physiol. Mensch. und Thiere, **29:** 13–40.

Popoff, N.

'09 L'ovule mâle et le tissu interstitiel du testicule chez les animaux et chez l'homme. Arch. Biol., **24:** 433–500.

Spengel, J. W.

'84 Hermaphroditismus bei Amphibien. Biol. Centralbl., **4:** 268–270.

Stohler, R.

'28 Cytologische Untersuchungen an den Keimdrüsen mitteleuropäischer Kröten. (*Bufo viridis* Laur., *B. calamita* Laur., *B. vulgaris* Laur.). Zeitschr. f. Zellf. und mikr. Anat., **7**: 400–475.

Sweet, G.

'08 Variation in the Anatomy of *Hyla aurea*. Proc. Roy. Soc. Victoria, **21**: 349–364.

Swingle, W. W.

'17 The Accessory Chromosome in a Frog Possessing Marked Hermaphroditic Tendencies. Biol. Bull., **33**: 70–79.

'26 The Germ Cells of Anurans. II. An Embryological Study of Sex Differentiation in *Rana catesbiana*. Journ. Morph. and Physiol., **41**: 441–546.

Takahashi, N.

'15 Ueber die Geschlechtsdrüsen der Wirbeltiere. I. Mitteil. Mitteil. med. Gesellsch. zu Tokio, **29**: 1–4.

'19 Ueber die Geschlechtsdrüsen der Wirbeltiere. II. Der wahre Hermaphroditismus der Fische und der Amphibien. Mitteil. aus med. Fak. der Kaiser. Univ. zu Tokyo, **22**: 287–320.

Witschi, E.

'14 Experimentelle Untersuchungen über die Entwicklungsgeschichte der Keimdrüsen von *Rana temporaria*. Arch. f. mikr. Anat., **85**: 9–113.

'21 Der Hermaphrodismus der Frösche und seine Bedeutung für das Geschlechtsproblem und die Lehre von der inneren Sekretion der Keimdrüsen. Arch. f. Entw.-Mech. der Organ., **49**: 316–358.

'23 Ueber die genetische Konstitution der Froschzwitter. Biol. Zentralbl., **43**: 83–96.

'27 Sex-Reversal in Parabiotic Twins of the American Wood-Frog. Biol. Bull., **52**: 137–146.

'29*a* Studies on Sex Differentiation and Sex Determination in Amphibians. II. Sex Reversal in Female Tadpoles of *Rana sylvatica* Following the Application of High Temperature. Journ. Exper. Zoöl., **52**: 267–292.

'29*b* Studies on Sex Differentation and Sex Determination in Amphibians. III. Rudimentary Hermaphroditism and Y Chromosome in *Rana temporaria*. Journ. Exper. Zoöl., **54**: 157–224.

Woronzowa, M. A.

'26 Fall von Hermaphroditismus bei *Rana temporaria*. Trudy Laboratorii Eksperimental'noj Biologii Moskovskogo Zooparks, **2**: 347–351.

INITIATION OF DEVELOPMENT IN ARBACIA.
VI. THE EFFECT OF SEA-WATER PRECIPITATES WITH SPECIAL REFERENCE TO THE NATURE OF LIPOLYSIN.[1]

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In 1910 Loeb reported that uninseminated eggs of the California sea-urchin, *Strongylocentrotus purpuratus*, form membranes while in solutions of $SrCl_2$ or $BaCl_2$. Because of the heavy precipitate of $BaSO_4$ which forms on the addition of the latter salt to sea-water, Loeb preferred to use the former. Loeb's work with these salts was done in connection with his experiments on the "fertilizing" effect of foreign blood and foreign cell extracts. Since this discovery, other workers have investigated the effects of foreign sera, treated in various ways. as agents for initiating development in echinid ova. The present writer also has made a similar study. He has likewise made a study of the effects of precipitates formed in sea-water after the addition of various substances to sea-water *alone*, in causing parthenogenetic development of *Arbacia* eggs. It is the latter study which forms the subject of the present communication. The results of the former, however, are also briefly reported. The work was begun at the Marine Biological Laboratory during the summer of 1919 and extended during several later years. Some of the precipitates were prepared not only during the summer months but also in April (three different years), in May (four different years), and in September (three different years)—*i.e.*, several weeks before the beginning and in the last days of the breeding season of *Arbacia*. In order to obtain results as far as possible beyond question, I repeated all of the experiments of previous years during the season of 1928. The data here given represent those collected during that year.

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[1] From the Marine Biological Laboratory, Woods Hole, Mass., and the Department of Zoölogy, Howard University, Washington, D. C.

The ox serum preparations first used were those made by my former students in the Department of Physiology of the Medical School of Howard University.

I may say at once that effects obtained by others with precipitates from sea-water containing organic matter and attributed to the presence of such matter, I have obtained with precipitates *from sea-water alone*. In what now follows, therefore, I present evidence to establish the thesis that these are hypertonic effects. It thus follows that theories of fertilization based on the effects of alleged substances, procured by treatment of sea-water containing organic or other foreign matter with various reagents, in causing parthenogenetic development of echinoderm ova, call for a careful scrutiny—if not total rejection.

The Experiments.

Loeb's discovery that $BaCl_2$ added to sea-water will cause the separation of the vitelline membrane in the uninseminated egg of *Strongylocentrotus purpuratus* may serve as our point of departure. In experiments on the uninseminated eggs of *Arbacia* I found $BaCl_2$ very toxic in the concentrations employed. I began, following Loeb, with a $\frac{3}{8}$ grammolecular solution.

$BaCl_2$.

Varying quantities of $\frac{3}{8}$ M or a 7 per cent. solution of $BaCl_2$ were added to egg suspensions. Drops of the eggs were removed from the $BaCl_2$-sea-water to 250 cc. of sea-water at half hour intervals during twelve to sixteen hours, and their development noted. During this period of time, samples of the eggs in the solutions were examined and the number of eggs with separated membranes counted. I have never found a cleaving egg either in the solution or in the sea-water to which I removed the eggs. I did find eggs with separated membranes and with thickened cortices. The eggs treated with $BaCl_2$ tend to clump in masses of the precipitate thrown down when $BaCl_2$ solution is added to sea-water, and they cytolyze in numbers. Twelve to sixteen hours after treatment with $BaCl_2$ many eggs are almost perfectly transparent, show their pigment aggregated in one mass, or form an extrusion of clear protoplasm. The eggs also fragment into spheres as small as the blastomeres of a normal egg in the thirty-two cell stage. These phenomena are also observed in eggs that are kept in the $BaCl_2$ solutions.

$BaCl_2$ plus Na_2SO_4.

For the experiments on the effects of $BaCl_2$ plus Na_2SO_4 in initiating development of uninseminated *Arbacia* eggs, two methods were used to prepare the activating agent. (1) To $BaCl_2$ solution plus sea-water Na_2SO_4 was added in excess several hours after the $BaSO_4$ precipitate had been completely thrown down. The clear supernatant solution, carefully decanted, was used as the activating agent. (2) After the addition of $BaCl_2$ solution to sea-water, the clear supernatant solution above the precipitate was discarded; to the precipitate treated with M/10 HCL Na_2SO_4 was added to excess. Here again, the clear supernatant solution was used as the activating agent. As in the work with $BaCl_2$ alone, a ⅜ M solution was used in varying proportions with sea-water. However, for reasons that will be made apparent beyond, in the majority of the experiments, a 7 per cent. solution of the salt was preferred. In all experiments made with this strength of $BaCl_2$, 1 part of the solution was added to 2 parts sea-water.

TABLE I.

EFFECT ON UNINSEMINATED *Arbacia* EGGS OF A 90 MINUTE EXPOSURE TO "$BaCl_2$-Na_2SO_4-SEA-WATER."

No.	1	2	3	4	5	6	7	8	9	10	11	12
Per cent. of cleavage	40	54	64	48	17	67	0	46	37	48	0	2
Per cent. of monaster and cytaster eggs	35	18	32	18	63	33	0	0	0	0	0	0
Per cent. of undifferentiated eggs	10	2	2	4	11	0	0	0	0	0	0	0
Per cent. of cytolyzed eggs 3 hours after exposure	15	26	0	30	9	0	100	54	63	52	100	98
"Swimmers" (20 hours after treatment)	++	++	+	+	++	+	0	+	+	+	0	0
Controls: uninseminated eggs in sea-water. Per cent. of membranes, cleavage, and larvæ	0	0	0	0	0	0	0	0	0	0	0	0

Table I gives the results of 12 experiments on the eggs—from 12 different females—whose fertilizable condition was known by the following tests: 1, speed and strength of the sperm agglutinating power of the egg sea-water; 2, rapidity and quality of membrane separation; 3, uniformity and rate as well as per cent. of cleavage; 4, viability of the plutei. That is, before an experiment was made Nos. 1 and 2 were determined. During its course Nos. 3 and 4 were determined. Elsewhere I have discussed the importance of certain cortical reactions as criteria for optimum fertilization capacity. The reader is referred to this paper for further details (Just, 1928). I now never make an experiment with eggs before testing them to determine their cortical responses to insemination. And whenever these are optimum I find that the eggs give close to 100 per cent. cleavage at an almost uniform rate; the larvæ are always vigorous.

Table I reveals that "$BaCl_2$-Na_2SO_4-sea-water" is an efficient parthenogenetic agent. The "swimmers" were merely estimated, not counted, since the amount of cytolysis after twenty hours is so great that counts are meaningless.

Since it seemed from these experiments made by treating the supernatant "$BaCl_2$-sea-water" with excess of Na_2SO_4 that the parthenogenetic agent is Na_2SO_4, I studied the effects of this salt added in excess to sea-water. I made this experiment many times during 1928. The appended table (Table II) of twenty-two experiments reveals that Na_2SO_4 in sea-water will initiate development. In these experiments the exposures varied from 40 to 90 minutes. I therefore made eight experiments in each of which I removed the eggs from the "Na_2SO_4-sea-water" to 250 cc. of sea-water after 30, 60, 90 minutes' exposure. The results are tabulated in Table III as Lot *A* (30 minutes' exposure), Lot *B* (60 minutes'), and Lot *C* (90 minutes'). It is at once apparent that there is a great deal of variation in the response of the eggs, as measured by the per cent. of cleavage and the production of swimmers, which cannot be clearly correlated with the length of exposure. It is also apparent, however, judging by the per cent. of cytolysis, that the shorter exposure is least harmful. Indeed, this is the case. While in the "Na_2SO_4-sea-water," the eggs show membranes after ten to fifteen

TABLE II.

EFFECT ON UNINSEMINATED *Arbacia* EGGS OF 40 TO 90 MINUTES' EXPOSURE TO "Na_2SO_4-SEA-WATER."

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Per cent. of cleavage	61	50	16	96	11	21	91	0	36	59	63	46	64	61	62	37	81	33	24	64	12	91
Per cent. of monaster and cytaster eggs	22	38	80	4	85	79	6	0	0	25	27	36	27	37	28	56	16	54	53	33	15	1
Per cent. of undifferentiated eggs	13	6	0	0	1	0	2	0	0	0	1	12	0	0	5	0	0	0	0	0	0	1
Per cent. of cytolysis 3 hours after exposure	3	6	4	0	3	0	1	100	64	16	9	6	9	2	5	7	3	13	23	3	73	7
"Swimmers" (24 hours after exposure)	+	++	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	0	0	+	0	++
Controls: uninseminated eggs in sea-water. Per cent. of membranes, cleavage, and larvæ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

minutes' exposure. Double this length of time, twenty to thirty minutes, is undoubtedly near the optimum exposure. The cleavages exhibited and the swimming forms produced by eggs so treated far more closely resemble those in the normal development of fertilized eggs. On the whole I consider Na_2SO_4 in sea-water an effective parthenogenetic agent.

Table III.

Effect of 30 (Lot *A*), 60 (Lot *B*), and 90 (Lot *C*) Minutes' Exposure to "Na_2SO_4-Sea-water."

Lot A.

No.	1	2	3	4	5	6	7	8
Per cent. of cleavage	47	44	63	55	51	25	66	48
Per cent. of monaster and cytaster eggs	47	47	37	43	44	69	34	48
Per cent. of undifferentiated eggs	0	0	0	0	0	0	0	0
Per cent. of cytolyzed eggs 3 hours after exposure	6	6	0	2	5	6	0	4
"Swimmers" (20 hours later)	+++	+++	++	+	++	+	+++	++++

Lot B.

Per cent. of cleavage	54	89	92	88	37	65	57	59
Per cent. of monaster and cytaster eggs	36	6	4	10	41	23	25	39
Per cent. of undifferentiated eggs	0	0	0	0	0	0	0	0
Per cent. of cytolyzed eggs 3 hours after exposure	10	5	4	2	22	12	8	3
"Swimmers" (20 hours later)	0	++	+++	++	0	+	+	+

Lot C.

Per cent. of cleavage	59	85	87	86	15	67	56	14
Per cent. of monaster and cytaster eggs	30	8	9	10	26	18	12	80
Per cent. of undifferentiated eggs	0	0	0	0	0	0	0	0
Per cent. of cytolyzed eggs 3 hours after exposure	11	7	4	4	59	15	32	6
"Swimmers" (20 hours later)	0	+	++	++	0	+	++	+
Controls: uninseminated eggs in sea-water. Per cent. of membranes, cleavage, and larvæ	0	0	0	0	0	0	0	0

The Precipitate Obtained from "Na_2SO_4-Sea-water" after the Addition of Acetone.

If to the supernatant solution drawn from "Na_2SO_4-sea-water" acetone be added, a voluminous precipitate forms. This precipitate dissolved in sea-water is a parthenogenetic agent. Sixteen experiments are cited.

In six experiments the procedure was as follows: To 200 cc. of sea-water Na_2SO_4 was added to excess. 60 cc. of the clear supernatant solution was carefully decanted and to it 300 cc. of acetone added. The voluminous precipitate was then thoroughly dried and dissolved in 70 cc. of sea-water.

1 cc. of eggs was removed from the eggs (of a single female), which had been allowed to settle in 250 cc. of sea-water, to 10 cc. of sea-water in which the acetone precipitate was dissolved. The results are tabulated in Table IV.

Table IV.

Effect on Uninseminated *Arbacia* Eggs of a 60 Minutes' Exposure to a "Na_2SO_4-Sea-water" Acetone Precipitate Dissolved in Sea-water.

No.	1	2	3	4	5	6
Cleavage	27	13	92	76	71	64
Monasters and cytasters	0	0	1	0	0	10
Undifferentiated	0	0	0	0	0	0
Cytolysis	73	87	7	24	29	26
Swimmers after 20 hours	0	0	0	0	0	0
Controls: uninseminated eggs in sea-water. Per cent. of membranes, cleavage, and larvæ	0	0	0	0	0	0

Ten other experiments were made in this wise:

To 200 cc. of sea-water, Na_2SO_4 was added to excess. 60 cc. of the clear supernatant solution was carefully decanted and to it 250 cc. of acetone were added. The heavy precipitate was then thoroughly dried for 48 hours and dissolved in 200 cc. of sea-water. As before 1 cc. of eggs (of a single female) was removed from 250 cc. of sea-water to 20 cc. of sea-water in which the acetone precipitate was dissolved. At 30, 60, and 90 minutes later samples of the eggs were removed to 250 cc. of sea-water. The results are tabulated—Lot *A* is the 30 minutes' exposure; Lot *B*, the 60 minutes'; and Lot *C*. the 90 minutes'—in Table V.

TABLE V.

EFFECT ON UNINSEMINATED *Arbacia* EGGS OF A 30, 60, AND 90 MINUTES' EXPOSURE TO "Na_2SO_4-SEA-WATER" ACETONE PRECIPITATE DISSOLVED IN SEA-WATER.

Lot A. 30 minutes' exposure.

No.	1	2	3	4	5	6	7	8	9	10
Cleavage	24	30	1	31	9	74	9	6	22	4
Monasters and cytasters	73	68	97	68	81	25	87	91	74	89
Undifferentiated	2	2	0	0	10	0	0	0	0	0
Cytolysis 2 hours after return to sea-water	1	0	2	1	0	1	4	3	4	7
"Swimmers" 20 hours later	0	0	0	0	0	0	0	0	0	0

Lot B. 60 minutes' exposure.

Cleavage	62	86	36	24	72	79	94	90	76	56
Monasters and cytasters	32	4	53	70	22	7	2	4	18	7
Undifferentiated	0	0	0	0	0	0	0	0	0	0
Cytolysis 2 hours after return to sea-water	6	10	11	2	6	4	4	6	6	37
"Swimmers" 20 hours later										

Lot C. 90 minutes' exposure.

Cleavage	82	38	10	32	52	15	24	15	17	12
Monasters and cytasters	12	39	2	58	7	1	1	0	2	1
Undifferentiated	0	0	0	0	0	0	0	0	0	0
Cytolysis 2 hours after return to sea-water	6	23	88	10	36	84	75	85	81	87
"Swimmers" 20 hours later	++ +++	+	0	++	++	+++ ++	++	+	+++	+++
Controls: uninseminated eggs in sea-water. Per cent. of membranes, cleavage, and larvæ	0	0	0	0	0	0	0	0	0	0

THE PRECIPITATE OBTAINED FROM "$BaCl_2$-Na_2SO_4-SEA-WATER" AFTER THE ADDITION OF ACETONE.

Experiments were also made with the precipitate obtained by adding acetone to the "$BaCl_2$-Na_2SO_4-sea-water." The first method of preparation employed was as follows:

200 cc. of clean filtered sea-water and 100 cc. of 7 per cent. $BaCl_2$ were kept for two hours with frequent stirring at 37° C. The suspension was then centrifuged and the supernatant solution discarded. The precipitate, after washing with $BaCl_2$, was

treated with N/10 HCl. To this was added Na_2SO_4 in excess and the mixture centrifuged. The clear supernatant solution was decanted and to it were added four volumes of acetone. This was then filtered and the precipitate washed with three changes each of absolute alcohol and of ether. It was dried for 48 hours before the addition of sea-water.

The second method employed follows:

Table VI.

Effect on Uninseminated *Arbacia* Eggs of a 30 and 60 Minutes' Exposure to "$BaCl_2$-Na_2SO_4-Sea-water" Acetone Precipitate Dissolved in Sea-water.

Lot A. 30 minutes' exposure.

No.	1	2	3	4	5	6	7	8	9	10
Cleavage	49	59	82	62	66	92	85	26	66	77
Monasters and cytasters	37	22	10	11	9	7	8	40	26	10
Undifferentiated	0	0	0	0	0	0	0	0	0	0
Cytolysis 2 hours after return to sea-water	14	19	8	27	25	1	7	34	8	13
"Swimmers" 20 hours later	+	+	+++	+	++	++++	+	+	+	++

Lot B. 60 minutes' exposure.

Cleavage	20	15	59	10	5	91	35	35	34	40
Monasters and cytasters	0	0	0	0	0	1	0	2	2	0
Undifferentiated	0	0	0	0	0	0	0	0	0	0
Cytolysis 2 hours after return to sea-water	80	85	41	90	95	8	65	63	64	60
"Swimmers" 20 hours later	+	+	0	+	+	+	+	0	0	+
Controls: uninseminated eggs in sea-water. Per cent. of membranes, cleavage, and larvæ	0	0	0	0	0	0	0	0	0	0

To 200 cc. of clean filtered sea-water 100 cc. of a 7 per cent. solution of $BaCl_2$ were added. After 24 hours the clear supernatant solution was decanted and Na_2SO_4 added to excess. This in turn was decanted and acetone added to the clear solution. The ten experiments, given in the table appended (Table VI), were made with this method, the results of the first method being reserved for mention beyond. In these experiments eggs were removed from the solution after 30 (Lot *A*), 60 (Lot *B*), and 90 (Lot *C*) minutes to normal sea-water. In all cases, however, ninety minutes' exposure resulted in their complete cytolysis, as revealed by careful examination two hours after their return to normal sea-water.

The Precipitate Obtained from Sea-water after the Addition of Acetone.

If acetone be added to clean filtered sea-water in the proportions, 1000 cc. of acetone to 250 cc. of sea-water, a flocculent precipitate is formed. This precipitate after thorough drying for twenty-four or more hours when suspended in 20 cc. of sea-water has feeble parthenogenetic power. For example, in ten experiments one drop of eggs was exposed to the action in 2 cc. of the acetone-precipitate-sea-water in tightly stoppered vials for two hours with the following results:

No. 1—0 per cent. cleavage; 31 per cent. cytolysis; 69 per cent. intact.
No. 2—2 per cent. cleavage; 84 per cent. cytolysis; 14 per cent. intact.
No. 3—0 per cent. cleavage; 38 per cent. monasters; 62 per cent. intact.
No. 4 .... 12 per cent. cytolysis; 88 per cent. intact.
No. 5—2 per cent. cleavage; .... 98 per cent. intact.
No. 6 .... 50 per cent. cytolysis; 50 per cent. intact.
No. 7 .... 10 per cent. cytolysis; 90 per cent. intact.
No. 8—1 per cent. cleavage; .... 99 per cent. intact.
No. 9 .... 100 per cent. intact.
No. 10—1 per cent. cleavage; 99 per cent. monasters.

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In other experiments, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 cc. of sea-water were added to the dried acetone-sea-water-precipitate. From these mixtures 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 cc. were taken as the parthenogenetic agent. The action of the acetone precipitate obtained from normal sea-water is at best feeble. But I have never failed to obtain the precipitate when acetone is added to sea-water; six different "C. P." preparations of acetone gave the same results. And at Naples, I obtained this precipitate with the use of ordinary "commercial" acetone.

The Precipitate Obtained from Sea-water after the Addition of 95 per cent. of Absolute Alcohol.

Beyond demonstrating that on addition of 95 per cent. of absolute alcohol to sea-water a flocculent precipitate is formed, I have not proceeded farther because of the high cost of alcohol.

I nevertheless attach importance to this fact. During the last six seasons at Woods Hole I have repeatedly demonstrated to fellow workers the action of both acetone and alcohol in throwing down a precipitate when either is added to sea-water.

Thrombin from the Serum of Ox Blood.

Thrombin from the serum of ox blood prepared either by the Schmidt or the Howell method when thoroughly dried exerts a feeble effect on the uninseminated eggs of *Arbacia*. This effect is revealed by cortical changes in the eggs and the production of a small per cent. of monasters, cleavage, and abnormal swimmers. Though the experiments were made during several years, I do not regard them worthy of more than passing notice at this point.

Hirudin and Curare.

A sample of "pre-war" hirudin in sea-water caused slight cortical changes in uninseminated *Arbacia* eggs with a small per cent. of cleavages and less than 0.1 per cent. swimmers. Suspensions of curare in sea-water were found less effective.

Discussion.

In 1918, Woodward reporting certain results obtained with precipitates from the "egg-sea-water" of *Arbacia* after its treatment with certain reagents, claimed the discovery of a parthenogenetic agent elaborated by the uninseminated eggs themselves and escaping into the surrounding sea-water. This agent Woodward named lipolysin. Her method of preparation is given in the following quotation.

"Precipitation of a lipolysin. Since there were strong indications of the presence of lipase and perhaps other enzymes, I adapted a method used by Robertson ('12) to obtain an enzyme from blood serum. Eight volumes of fertilizin and four volumes of 7 per cent. $BaCl_2$ were kept at 37° C. for an hour or more and stirred frequently. The mixture was then centrifuged and the filtrate discarded. The precipitate was washed several times with $BaCl_2$ and then treated with N/10 HCl. To this solution was added Na_2SO_4 in excess to precipitate the barium, and the mixture allowed to stand overnight. The liquid was then centrifuged and to the clear fluid were added four to five volumes of acetone, which caused a heavy flocculent precipitate. This was filtered, and the precipitate was washed several times with absolute alcohol and ether, and then dried for thirty-six hours or more over H_2SO_4. The resulting powder dissolves readily in both sea-water and distilled water. It should be added that this precipitate was invariably obtained with good fertilizin and freshly distilled acetone during the summer of 1915 and until the middle of July, 1916. It was never obtained by adding acetone to sea-water. By the middle of 1916, all of the recently purchased acetone had been used up, and recourse was necessary to some purchased from Kahlbaum in 1912. This was yellowish in color, slightly acid to litmus, and differed slightly in odor from the fresh. With this acetone, no precipitate was obtained excepting after adding NaOH, and even then only in traces." (Woodward, 1918, pages 475–476.)

Using normal sea-water instead of "egg sea-water," I have followed Woodward's method exactly. On obtaining positive results—even better than those she recorded—I next analyzed separately the parthenogenetic action of each reagent she employed. The experiments detailed above constitute the evidence for the statement that the use of egg-sea-water for the precipitation of lipolysin is wholly superfluous; this "lipolysin" is present in normal sea-water after its treatment with any one or all of the reagents in Woodward's method. The alleged "lipolysin" is merely hypertonic sea-water. Let us summarize the results which I have given above:

1. $BaCl_2$ as Loeb showed years ago for eggs of *S. purpuratus* has a feeble parthenogenetic effect. This is likewise true for the uninseminated eggs of *Arbacia*.

2. Either the precipitate formed after the addition of $BaCl_2$ to normal sea-water when treated, as Woodward treated "egg-sea-water," with Na_2SO_4 or the solution above the $BaSO_4$ precipitate in sea-water on the addition of Na_2SO_4 to excess is a good parthenogenetic agent.

3. Na_2SO_4 added to normal sea-water in excess is an effective parthenogenetic agent.

4. The precipitate obtained after the addition of acetone

to "$BaCl_2$-sea-water" or to "$BaCl_2$-Na_2SO_4-sea-water" brings about parthenogenetic development. The washed, HCl-treated precipitate from normal sea-water thrown down by $BaCl_2$ after the addition of Na_2SO_4 to excess has a similar effect. It did not seem worthwhile to give above the details of this latter finding.

Consideration of these four lines of evidence leads one to the conclusion that the effects are due to hypertonicity and not to the presence of a lipolysin. Simply the results with Na_2SO_4 in normal sea-water acting alone are fatal to Woodward's theory of the initiation of development. Moreover, the experiments made to learn the effects of the precipitate formed on the addition of acetone to normal sea-water, if the four lines of evidence summarized were wanting, would go a long way toward discrediting Woodward's theory. She says that she never obtained a precipitate by adding acetone to sea-water. With both "c.p." acetone of all manufacture available and commercial acetone, as well as with ninety-five per cent. and absolute alcohol, I have never failed to get a voluminous flocculent precipitate from normal sea-water. Even if this precipitate had no effects on the uninseminated eggs, the fact that it forms under these conditions is alone a serious obstacle to the acceptance of Woodward's work.

Now I have obtained precipitates from "Na_2SO_4-sea-water" and "$BaCl_2$-Na_2SO_4-sea-water" after the addition of acetone as early as April, weeks before the breeding season of *Arbacia* begins. Again, in the last days of September, when ripe eggs of *Arbacia* are scarce or indeed unobtainable, have I obtained the precipitate. Surely, there could scarcely be available during these months any lipolysin in the normal sea-water around Woods Hole!

The objections against the lipolysin theory thus appear, to me at least, far too serious to warrant its acceptance.[1] Moreover, built in a measure on Glaser's theory of auto-parthenogenesis, which itself is open to grave suspicion (see Just, '28 *b*), it can only deserve a hearing by the establishment of wholesale errors in this present communication.

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[1] It is extremely doubtful, for example, that according to her own description, Miss Woodward ever observed agglutination of *Asterias* sperm by *Asterias* egg-water. According to her description, if a drop of egg-water was "injected under a cover-glass into a drop of sperm suspension, the sperm would gather into small irregular angular clusters of six to eighteen and remain agglutinated for a number of seconds." This is certainly not comparable to agglutination in *Arbacia, Echina-*

I have elsewhere pointed out grave defects in the lipolysin theory: logically, it does not and cannot explain either experimental parthenogenesis or fertilization—even if its experimental basis be correct. More, a mere superficial reading of Woodward's paper reveals it to the reader as a mass of contradictions. Both on grounds of experiment and on grounds of logic, therefore, the lipolysin theory is lacking.

The lipolysin theory thus belongs in the same category with Robertson's ovocytase. I believe that thrombin from the serum of ox blood, prepared by either the method of Schmidt or that of Howell is as effective as Robertson's so-called ovocytase. That it would be dangerous to theorize on this point, one may conclude from the effect of hirudin. Thrombin *per se* has no particular virtue if it be true that hirudin likewise—like a host of foreign substances—effects changes in the egg of *Arbacia*. Robertson's work too generally has been allowed to go unchallenged. It is work like this that needlessly cumbers progress in the field of fertilization.

The purpose of this first work (1914) was to study the effect of thrombin as an activating agent. I was led to this work

rachnius, or *Nereis*. Lillie has repeatedly failed to obtain agglutination in *Asterias*. Glaser claims to have observed agglutination of starfish sperm but gives no details of his observations. On the other hand, using normally shed eggs and sperm over a period of years, I have yet to observe agglutination of *Asterias* sperm by *Asterias* egg-sea-water.

It should be noted, however, that absence of a sperm agglutinin in *Asterias* egg-water is of no consequence for Miss Woodward's theory since she claims to have demonstrated that the sperm agglutinin is distinct from "Lipolysin" (478, 480, 487). It is the "lipolysin" alone that plays the leading rôle in the initiation of development. It would thus seem that any criticism of the lipolysin theory based on the absence of sperm agglutinin in *Asterias* egg-water is beside the point. But this is not wholly true, it would seem from Miss Woodward's own statements: "Lipolysin" is a lipolytic enzyme, possessed of parthenogenetic power, while the other separate entity, the sperm agglutinin, lacks it. Says Miss Woodward: "If the agglutinin is an enzyme, its nature is not yet known" (page 479), though the effect of x-radiation suggests this. One is thus led to infer that the effect of the x-radiation is on the enzyme, "lipolysin," which is present but not identical with the agglutinin. Its mere presence, however, is sufficient to make the agglutinin behave like an enzyme when subjected to x-radiation.

through reasoning somewhat like this: The fertilization reaction might be comparable to the action of thrombin in the coagulation of blood. There might thus be two possible modes of interpreting the reaction in a manner similar to two of the then existing theories of blood coagulation: First, the egg produces a substance which with calcium in the sea-water forms a substance; and it is this substance with which the sperm unites to bring about fertilization. Or, fertilization results when a substance, comparable to blood thrombin, is in excess of another substance, comparable to antithrombin, and with which the sperm unites.

Woodward's lipolysin theory of fertilization more closely resembles the latter scheme. Unfortunately, it seems to me, her work is open to question. Subsequent work convinced me that any notion of my own concerning the analogy between blood coagulation and fertilization is only superficial. The effects of thrombin, were they to induce one hundred per cent. development, could scarcely give support to this notion. And if they did, the effects of hirudin would outweigh it.

On the other hand, Lillie's fertilizin theory does resemble the following scheme for the explanation of the blood coagulation:

Cellular elements → thrombokinase
Thrombokinase + calcium + thrombogen = thrombin
Thrombin + fibrinogen = fibrinogen.

That is, according to the fertilizin theory, the fertilization-reaction might run thus:

Cellular elements (the eggs) → fertilizinkinase
Fertilizinkinase + calcium = fertilizin
Fertilizin + sperm-receptor = fertilization.

But this scheme at best is only a crude and superficial analogy. Moreover, after I had made experiments on the effects of oxalated sea-water (1915) and on the fertilization capacity of eggs in various salt solutions (referred to in Lillie and Just, 1924) I abandoned this notion. Even if the scheme for explaining blood coagulation given above were correct, it would be hazardous to compare this phenomenon with fertilization. Lillie's original statement of his theory thus remains the best working hypothesis for the analysis of fertilization. Fertilization is a simple two-body reaction—a reaction between an egg substance, fertilizin, and the spermatozoön.

I have repeatedly emphasized the necessity of analyzing the fertilization-reaction in terms of the behavior of the gametes themselves in the presence of each other. The work on experimental parthenogenesis makes up a brilliant chapter in modern biology; but experimental parthenogenesis is more significant for the analysis of cell division than for that of the reaction between gametes. Agents of experimental parthenogenesis from the very nature of the case can at best never fully bring about in the egg those changes initiated by insemination. Consider for a moment the results, which I have recently summarized (1928 *a*), obtained with various agents that induce membrane separation in *Arbacia* eggs. These agents, if they do not call forth the optimum response by the eggs, do too little, and the eggs show no change; or they do too much, and the eggs cytolyze. What is more important, however, is that eggs with membranes separated by the action of experimental agents *are not in the same condition as eggs with membranes induced by insemination.* The basis for this statement is the fact that eggs with membranes separated by such agents do not respond with the formation of extra-ovates when placed in distilled water; on the other hand, eggs with membranes separated after insemination do form extra-ovates. The majority of the agents used for experimental membrane separation are presumably effective over the whole surface; the spermatozoön is rapidly effective within an exceedingly narrow cortical *locale.* Again, with the best methods for experimental parthenogenesis eggs often fail to respond though they are capable of fertilization. Finally, the per cent., normality, and viability of the development induced by experimental agents under the best conditions frequently fall short of those which result from insemination.

If these statements be true, it must follow that physico-chemical explanations of fertilization though based on refined and technically excellent physico-chemical work on experimental parthenogenesis demand careful criticism. It is my judgment that unless agents of experimental parthenogenesis exactly duplicate the morphology (or biology) of fertilization in every egg exposed, the interpretations by the modernistic school of physico-, chemico-, and mathematico-biologists of fertilization in terms of experimental parthenogenesis rest on insecure grounds. For example, the measurement of the oxygen consumption, the CO_2 output, the viscosity changes, and the whole category of physico-chemical changes exhibited by eggs with membranes separated after treatment with agents that cause the eggs to go no farther in their development are exceedingly important. Thus, also, similar measurements on eggs so treated (by hypertonic sea-water, for instance) that they develop without the cortical responses typical of inseminated eggs. But in neither case, however, are we dealing with responses exactly similar to those brought about by insemination. The present status of the fertilization problem, therefore, demands a rigorous *biological* analysis of the fertilization-reaction itself, before we can venture a physico-chemical approach to our problem. In addition, finally, we must appreciate the limited applications of the work on experimental parthenogenesis to fertilization.

LITERATURE CITED.

Glaser, Otto.
'14 On Auto-parthenogenesis in *Arbacia* and *Asterias*. Biol. Bull., **26**: 387–409.

Just, E. E.
'22 The Effect of Sperm Boiled in Oxalated Sea-water in Initiating Development. Science, **56**: 202–204.
'28*a* Initiation of Development in *Arbacia*. IV. Some Cortical Reactions as Criteria for Optimum Fertilization Capacity and Their Significance for the Physiology of Development. Protoplasma, Bd. V, 97–126.
'28*b* Initiation of Development in *Arbacia*. V. The Effect of Slowly Evaporating Sea-water and Its Significance for the Theory of Auto-Parthenogenesis. Biol. Bull., **55**: 358–367.

Lillie, Frank R.
'14 Studies of Fertilization. VI. The Mechanism of Fertilization in *Arbacia*. Jour. Exp. Zoöl., **16**: 523–590.

Lillie, F. R., and Just, E. E.
'24 Fertilization. In General Cytology, University of Chicago Press.

Loeb, J.
'10 Die Sensitivierung der Seeigeleier mittelst Strontiumchlorid gegen die entwicklungserregende Wirkung von Zellextracten. Archiv. f. Entwicklungs-Mechanik, XXX, 44.

Robertson, T. B.
'12 On the Isolation of Oöcytase, the Fertilizing and Cytolyzing Substance in Mammalian Blood Sera. Jour. Biol. Chem., Vol. 11, pp. 339–346.

Woodward, Alvalyn E.
'18 Studies on the Physiological Significance of Certain Precipitates from the Egg Secretions of *Arbacia* and *Asterias*. Jour. Exp. Zoöl., **26**: 459–501.

EFFECTS OF LOW TEMPERATURE ON FERTILIZATION AND DEVELOPMENT IN THE EGG OF *PLATYNEREIS MEGALOPS*.[1]

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Having previously learned that low temperature induces a per cent. of the eggs of both *Nereis* and *Platynereis* to develop after insemination without the extrusion of the polar bodies, I ran some additional experiments on the eggs of the latter during the summer of 1927. This I did because the nuclear phenomena show so clearly in the living egg. I therefore hoped to be able to isolate, just before first cleavage, eggs with quintuple haploid nuclei for the purpose of rearing them to sexual maturity. Eggs of *Platynereis* were chosen because I have had a greater degree of success in rearing them than those of *Nereis*. The method of exposure to cold was preferred to that of ultra-violet radiation; since normal eggs obtained only after copulation of the worms are already inseminated and since exposure to ultra-violet up to five minutes is without effect on the eggs in a virgin female, uninseminated eggs cannot be subjected to radiation in sea-water as is the case with those of *Nereis*. The exposure of dry eggs, which are capable of normal fertilization and development, to ultra-violet radiation has not yet been undertaken.

The Experiments.

Two series of experiments were made: one on the effects of low temperature on uninseminated eggs in the females; and one on eggs normally laid.

Forty-eight females were placed in the cold chamber at a temperature of 3.5° C. After ten hours at this temperature they were removed. As soon as the water containing them reached the temperature of the sea-water in the laboratory and at intervals thereafter they were allowed to copulate each with one male from those kept over-night in normal sea-water. The per cent. of cleavage bore no constant relation to the time of copulation and egg-laying as the following tabulation reveals:

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[1] From the Marine Biological Laboratory, Woods Hole, Mass., and the Department of Zoölogy, Howard University, Washington, D. C.

No.	Time of Egg-laying.	Per cent. No Cleavage.	Per cent. Cleavage.
1	11:05 A.M.	100	0
2	11:10	100	0
3	11:11	44	56
4	11:12.5	100	0
5	11:15.5	100	0
6	11:17.5	97	3
7	11:22	100	0
8	11:24.5	100	0
9	11:26	74	26
10	11:28	100	0
11	11:32.5	100	0
12	11:38.5	91	9
13	11:41	50	50
14	11:44.5	100	0
15	11:53	18	82
16	11:54	68	32
17	11:55.5	100	0
18	11:58.5	74	26
19	12:02 P.M.	100	0
20	12:12	99	1
21	12:14	34	66
22	12:15	60	40
23	12:16.5	64	36
24	12:18	92	8
25	12:20	74	26
26	12:23	88	12
27	12:30	68	32
28	12:30	100	0
29	12:31	96	4
30	12:35.5	100	0
31	2:25.5	100	0
32	2:27	99	1
33	2:31.5	100	0
34	2:34.5	100	0
35	2:36	26	74
36	2:37	42	58
37	2:40	42	58
38	2:41	92	8
39	2:42.5	96	4
40	2:46	100	0
41	2:47	62	38
42	2:48.5	94	6
43	2:54	56	44
44	2:55	100	0

It would be erroneous to attribute these results entirely to the effects of low temperature for the reason that *Platynereis*, especially the males, are not good laboratory animals. That is, under laboratory conditions ideal for *Nereis*, for example, they early suffer a loss of vitality since animals kept over-night at room temperature never give normal fertilization and development.

The other series of observations was made on the development of eggs exposed to low temperature after egg-laying.

Twenty-five female *Platynereis* were allowed to copulate immediately after capture, each with a different male. Three minutes after laying, each lot of eggs was placed in the cold chamber at a temperature of 3.5° C. The next day (ten hours after egg-laying) the eggs were brought from the cold chamber and allowed to return gradually to room temperature without changing the sea-water. The results obtained are recorded as follows:

Female No.	Time of Egg-laying.	Per cent. Cleavage.	Per cent. "Swimmers."
1	10:39 P.M.	50	4
2	10:40	6	2
3	10:41	74	6
4	10:43.75	18	4
5	10:41.75	88	50
6	10:44	60	14
7	10:43.5	16	0
8	10:40.75	78	14
9	10:41.5	92	28
10	10:43.5	78	14
11	10:43	14	2
12	10:43.5	84	70
13	10:41.7	74	16
14	10:40.5	86	46
15	10:41	82	28
16	10:43	90	32
17	10:43.6	64	20
18	10:46	68	32
19	10:45	74	24
20	10:45	98	12
21	10:45	98	16
22	10:47	84	28

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Because the per cent. of first cleavage in many cases is difficult to ascertain, it is best to make counts when the majority of cells are in the eight cell stage; later, it is still easier to make the counts. The oil drops, especially in those eggs that fail to cleave, are abnormally reduced in number; many eggs show the oil drops coalesced into one. Many cells that do not show cleavage show nuclear divisions, even in cases where the oil drops are normal in number; such eggs differentiate as far as the swimming stage without cleavage.

Discussion.

The action of low temperature on eggs is by no means simple. This is especially true for the egg of *Platynereis*. However one of its effects on this egg is to inhibit the extrusion of polar bodies with or without subsequent normal development. The retention of the polar bodies within the cell brings about the formation of four maternal haploid nuclei with one, two, three, or all of which the sperm nucleus unites. This fact has been ascertained by study of the living egg; the phenomenon was (during 1926 and 1927) demonstrated to several fellow workers. Where, however, as in most cases, development is abnormal or fails we must not conclude that the results are due to polar body retention and the consequent presence of female nuclei above the orthodox single zygote nucleus.

In addition, the effect of low temperature presumably is not localized to any one area of the cortex; the whole cortex is affected. If this be true, it follows that the production of quintuple zygote nuclei is not due to localized injury. *Nereis* eggs inseminated after ultra-violet radiation reveal a localized cortical injury which endures through the stages of development including the trochophore. Ultra-violet radiation also causes alteration of the polarity of the egg. It may be that further comparative study of the effects of low temperature and of ultra-violet radiation on the eggs of these nereids may give us valuable information concerning the rôle of the cortex in determining polarity.

HYDRATION AND DEHYDRATION IN THE LIVING CELL. II. FERTILIZATION OF EGGS OF *ARBACIA* IN DILUTE SEA-WATER.[1]

E. E. JUST,

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Several years ago I made observations on the fertilization of eggs of *Echinarachnius parma* in dilute sea-water (Just, '23). These observations have now been extended to include the eggs of several forms (Just, '28). During the summer of 1927, working at the Marine Biological Laboratory, Woods Hole, Mass., I repeated some older observations on the fertilization capacity of eggs of *Arbacia* in dilute sea-water. These observations early established several interesting facts, among them: first, that in the greatest dilutions in which fertilization is possible, the time of first cleavage is delayed; secondly, that fertilization capacity in these dilutions depends upon the concentration of the egg suspensions—*i.e.*, it is greatest when the suspension is attenuated; thirdly, the agglutination of sperm is possible in dilute sea-water; and finally, that fertilization is possible in greater dilution than that in which eggs of *Echinarachnius* are capable of fertilization. The present communication, however, deals with the last-named finding only. The action of the dilutions employed is wholly reversible: eggs exposed to them for hours on return to normal sea-water fertilize and develop normally.

The Experiments.

Eggs of *Arbacia* fertilize readily in dilutions of 95, 90, 85, and 80 parts of sea-water plus 5, 10, 15, and 20 parts respectively of distilled water, as repeated experiments show. (Cf. Medes.) On ascertaining this fact I did not proceed farther since the object of my experiments was to learn the greatest degree of hypotonicity in which fertilization is possible. I therefore made a large number of experiments on the fertilization of eggs in the dilutions 75, 70, and 66⅔ parts sea-water plus 25, 30, and 33⅓ parts, respectively, of distilled water. The last named dilution is close to the limit for fertilization. A protocol of a typical experiment with 75 and 70 per cent. sea-water follows:

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[1] From the Marine Biological Laboratory, Woods Hole, Mass., and the Department of Zoölogy, Howard University, Washington, D. C.

July 7, 1927. 8:15 A.M. 3 females opened. Eggs from each to 250 cc. of sea-water. Each lot of eggs washed at 8:35, 9:08, and 9:16 A.M. At 10:10 A.M. eggs tested for fertilization capacity and the best lot retained. At 10:20 A.M., 3 graduates prepared as follows: No. 1, 100 cc. of sea-water plus 0 cc. of distilled water; No. 2, 75 cc. of sea-water plus 25 cc. of distilled water; No. 3, 70 cc. of sea-water plus 30 cc. of distilled water. At 10:28 A.M., 1 cc. of eggs added to each graduate. At 11:08 A.M., each lot of eggs poured into finger bowls and inseminated in the solutions.

12:02 P.M., first cleavage in Nos. 1 and 2; cleavage is indicated in some eggs of No. 3.

1:48 P.M., No. 1 (inseminated in sea-water), 98 per cent. cleavage—8- and 16-cell stages.

No. 2 (inseminated in 75 cc. of sea-water plus 25 cc. of distilled water), 90 per cent. cleavage.

No. 3 (inseminated in 70 cc. of sea-water plus 30 cc. of distilled water), 75 per cent. cleavage. Cleavages irregular and arrested; mostly 4- and 8-cell stages with some 3-cell stages.

6:45 P.M. All 3 dishes show good late cleavage stages. One swimming blastula in No. 1.

8:40 P.M. Normal embryos (No. 1) are swimming.

8:50 P.M. Very few "swimmers" in No. 2. No "swimmers" in No. 3.

9:47 P.M. All swimming in No. 2. About 3 per cent. swimming in No. 3; others, except those not fertilized, still in their membranes.

July 8, 7:30 A.M. Normally inseminated (No. 1) are now plutei. No. 2—50 per cent. are top swimming gastrulæ; 50 per cent. are rotating blastulæ on bottom of dish. No. 3. Great majority are abnormal blastulæ on bottom of dish away from the light; those that are suspended, early but extremely active gastrulæ.

Subsequently throughout the breeding season of *Arbacia* this experiment was repeated with the difference that greater dilutions of sea-water were used. For example, on July 8 in ten experiments with 66⅔ per cent. sea-water, the average per cent. of cleavage was 44. Of the eggs that failed to cleave, 18 per cent. showed separated membranes. In all these experiments with 70 and 66⅔ per cent. sea-water first cleavage took place later than in eggs inseminated in sea-water. Also, when these experiments were repeated daily for the next five days, it was discovered that a higher per cent. of cleavage was obtained in a given volume of dilute sea-water if only a few eggs were used. Experiments subsequently were therefore made always with 150 cc. of sea-water or given dilution to which was added *one drop* of eggs. The following is typical of a group of eight experiments:

July 16, 9:10 A.M. Each lot of eggs from nine females washed four times in 250 cc. of sea-water. Samples from each lot when tested at 11:00 A.M. show optimum fertilization reaction. 11:08 A.M., one drop of eggs from each of the nine lots added to 9 dishes containing each 150 cc. of sea-water (Series I).

Similarly one drop of eggs from each lot added to each of 9 dishes containing 100 cc. of sea-water plus 50 cc. of distilled water (Series II).

12:08 P.M., eggs of both series inseminated.

12:10–12:14 P.M. These eggs in normal sea-water show 98 to 100 per cent. beautiful membranes.

12:15 P.M., eggs of Series II (in 66⅔ per cent. sea-water) show following with respect to membranes:

No. 1—100 per cent. full membranes.
No. 2—2 per cent. full membranes, 9 per cent. tight membranes.
No. 3—50 per cent. full, 50 per cent. tight membranes.
No. 4—100 per cent. full membranes.
No. 5—94 per cent. full membranes, 4 per cent. tight membranes, 2 per cent. cytolysis.
No. 6—93 per cent. full membranes but thin; 7 per cent. tight membranes.
No. 8—98 per cent. tight membranes, 2 per cent. no fertilization.
No. 9—97 per cent. full membranes, 1 per cent. cytolyzed, and 2 per cent. fused eggs—tight membranes ruptured in each at point of fusion.

1:00 to 1:10 P.M. Samples of each of the nine lots inseminated in sea-water (Series I) show first cleavage—98 to 100 per cent.

1:35–1:45 P.M., these eggs in dilute sea-water show cleavage as follows:

No.	1	2	3	4	5	6	7	8	9
Per cent. of cleavage	70	65	33	64	89	30	45	43	42

Eggs of Series I are now beautiful perfectly normal top "swimmers."

Quite frequently as in the experiment now to be cited eggs were allowed to stand for several hours in sea-water. At the time of transfer to the dilution an equal bulk of eggs was placed in equivalent volumes of sea-water.

For example:

July 15, 3:40 P.M., to each of 9 dishes containing 100 cc. of sea-water plus 50 cc. of distilled water is added one drop of eggs that have been in sea-water since 9:10 A.M. (6½ hours). Eggs are similarly added to 9 dishes containing 150 cc. of sea-water.

4:45 P.M., both series of eggs are inseminated.

5:45–5:55 P.M. Eggs inseminated in sea-water give the following:

No.	1	2	3	4	5	6	7	8	9
Per cent. of cleavage	98	99	97	98	94	96	89	78	86

6:00 P.M. No cleavages among eggs in the solution.

6:30 P.M. Some eggs show cleavage beginning.

6:40 P.M. Eggs in 66⅔ per cent. sea-water give the following:

No.	1	2	3	4	5	6	7	8	9
Per cent. of cleavage	24	35	40	30	52	11	36	27	49

Whereas, however, on the next day (9:10 A.M.) the eggs inseminated in sea-water show percentages of gastrulæ almost identical to those for cleavage, these eggs in the 66⅔ per cent. sea-water give the following:

No.	1	2	3	4	5	6	7	8	9
Per cent. of blastulae	32	38	1	2	50	0	30	0	17

The counts did not include intact unfertilized eggs which means therefore that eggs if fertilized either developed or cytolyzed. Eggs in No. 9 showed many microblastulæ and twins.

Discussion.

The reversible effects of dilute sea-water or of other hypotonic solutions on the uninseminated echinid egg are too generally known to warrant lengthy discussion. That *Arbacia* eggs are capable of fertilization and development *in* a given dilution of sea-water would seem further to indicate that such dilution is not deleterious. This statement must not be taken to mean that the effects of a still greater dilution which prohibits fertilization are irreversible. However, claims that a given solution produces a reversible effect would be greatly strengthened by the simple procedure of testing the fertilization capacity of eggs which on removal from the solution have come into equilibrium with the normal sea-water.

The interpretations of the effects of hypotonic solutions on echinid ova—those of *Arbacia*, for example—are largely in terms of osmotic pressure. Now I have found, as others previously, for several ova, that sea-water of a dilution which permits fertilization the effects of which are reversible brings about the imbibition of water by the eggs while in the dilution; on their return to normal sea-water, *they show vacuoles in the cytoplasm which migrating to the egg surface disappear.* The uninseminated egg of *Arbacia* is not the most favorable object for the study of this phenomenon. Nevertheless, like the egg of *Nereis* (Just, '26), it does exhibit it. A theory of the permeability of the *Arbacia* egg to water should consider this phenomenon.

LITERATURE CITED.

Just, E. E.

'23 The Fertilization Reaction in *Echinarachnius parma.* VIII. Fertilization in Dilute Sea-water. Biol. Bull., Vol. XLIV.

'26 Artificial Production of Vacuoles in Living Cells. Anat. Rec., Vol. XXXIII.

'28 Hydration and Dehydration in the Living Cell. I. The Effect of Extreme Hypotony on the Egg of *Nereis.* Physiological Zoölogy, Vol. I, pp. 122–135.

Medes, G.

'17 A Study of the Causes and the Extent of Variations in the Larvae of *Arbacia punctulata.* Jour. of Morph., Vol. XXX, 317–432.

THE TOXICITY OF MONOVALENT AND DIVALENT CATIONS FOR SEA URCHIN EGGS.[1]

IRVINE H. PAGE.

The literature describing the action of salts on animal protoplasm (1–18), plant protoplasm (18–21), special physical systems (22–31) and the more specific case of the red blood cell seems to show that the toxicity of electrolytes is not of the same order for all types of cells. This fact renders it probable that protoplasm is not necessarily a "stuff" of uniform reactivity regardless of its origin.

"Salt toxicity" is probably the composite picture of many individual reactions. Salts have the most variant actions on lipoids, soaps, proteins, etc., and since protoplasm is made up of complex mixtures of these special moieties it is difficult to conceive in our present state of knowledge how we can ever predict "toxicity." This conception seems to have been overlooked in much of the investigative work concerning the toxicity of salts especially for highly differentiated organisms. Thus sodium might be very toxic for one tissue while nontoxic for another tissue and our term "toxicity," when applied to differentiated organisms, then becomes a miscellany of the greatest intricacy, on the one hand that of complex cellular reactivities and on the other complex cellular types.

Li and Na form one, and K, Rb, and Cs the other subdivision of the alkali metal period. If only the chemical structure and related physical properties of the reagent were to determine the physiological response of the protoplasm regardless of the protoplasmic type with which we deal we might anticipate that the relations which these metals maintain in the periodic system should be maintained in their action on protoplasm. The tendency should always be toward a grouping of Li and Na on the one hand and Rb, Cs, and K on the other. In addition Li and Mg should resemble Ca in some respects and Na in others.

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[1] Contribution from the Research Division of Eli Lilly and Company, Marine Biological Laboratory, Woods Hole, Mass.

Many types of protoplasm respond to salts in such a way as to suggest that the specific chemical nature of the salt is, indeed, largely responsible for its reactions. However one must still appreciate the fact that the reagent electrolyte is only one half of the reacting system; the sum total of the effect we call "salt toxicity."

Although an explanation of toxicity is far from forthcoming nevertheless the data on toxicity are important in order to correlate other vistas of research on protoplasm with this complex phenomenon.

METHODS.

The eggs of the Sea Urchin (*Arbacia punctulata*) were used in all the experiments. It may be stated at the outset that a possible source of error occurs in the resistance of individual eggs. However the difference observed in toxicity to the electrolytes are well beyond such experimental variations.

The eggs were removed directly from the ovaries with a pipette and placed in the isotonic solution of the purest electrolyte obtainable. Particular difficulty was experienced in obtaining a good quality $CaCl_2$. Freezing point determinations for Woods Hole sea water (G. Walden (32)) gave the following molar equivalents for the electrolytes. $NaCl = 0.52$, $KCL = 0.53$, $CaCl_2 = 0.34$, $MgCl_2 = 0.35$. The following are only approximate: $Na_2HPO_4 = 0.40$, $LiCl = 0.56$, $CsCl = 0.53$, $RbCl = 0.56$.

Method 1.—In order to measure the effect of salts on protoplasm it is obvious that the salt must be in contact in a pure condition with the eggs. For instance, a mere trace of Ca or Mg profoundly modifies the toxicity of the monovalent cations. It is for this reason that it was found necessary to centrifuge the eggs three times in a solution of electrolyte to be tested adding fresh electrolyte at each centrifuging. The eggs were then transferred to Stender dishes, and 5 c.c. of the suspension removed at certain intervals of time, placed in a large quantity of sea water, washed and fertilized. After 24 hours the percentage development was estimated.

Curiously enough it is important that the eggs be taken directly from the ovaries particularly when the divalent cations are used (Heilbrunn (33)) and Page (32a). Eggs which have been washed in sea water and allowed to stand, when placed in the electrolyte solution and centrifuged, quickly agglutinate. This has been shown by Chambers (34) to be due to the fact that $CaCl_2$ causes the jelly surrounding the egg to become very sticky after the eggs are returned to sea water whereas in KCl the jelly is simply dispersed. Due to the presence of $CaCl_2$ this adhesiveness causes the eggs to stick together so tightly that when centrifuged cytolysis occurs through mechanical tearing of the eggs. The same phenomenon occurs with NaCl but not in such a striking manner as with $CaCl_2$. Why fresh, unwashed eggs show much less tendency to clump on electrolyte treatment is difficult to say.

Method 2.[1]—Since it is possible that the relatively severe centrifugation employed in the first method might influence the results, we resorted to the following technique:

Eggs shed from the ovaries were very gently centrifuged in order to remove most of the sea water. Pyrex glass tubes 5 ft. long and ½ inch in diameter were filled with the electrolyte to be tested. The eggs were then mixed with a little of the salt solution and dropped into the top of the tube. As the eggs fell slowly through the solution a continuous washing process was in action. Convection and diffusion of the salts in the upper part of the tube into the lower part could hardly occur due to the length of the tubes and their relatively small bore. These washed eggs were then placed in large bowls of sea water, washed, and fertilized. At one and half and three hours, samples were removed and fixed in 3 drops of 20% formaldehyde to 3 c.c. of egg suspension. Counts were made in each sample, noting the number of eggs which were undivided and the number which were in each stage of division. By multiplying the numbers of two celled eggs by one, the four celled eggs by two, the eight celled by three, etc. and dividing the total number of divisions by the total number of eggs, the number of divisions per egg was determined. This figure was then corrected by the control (equalled 100%) which had been subjected to the same handling in sea waer. The temperature of the sea water varied between 20–23 degrees C. The $[H^+]$ of the electrolytes used as determined colorimetrically with the

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[1] Our thanks are due Miss Kellicott for her work during this part of the investigation.

Clark and Lubs indicator series were $NaCl = 6.3$, $KCl = 6.4$, $CaCl_2 = 7.6$, $MgCl_2 = 6.3$.

RESULTS.

Figure 1 shows the results of treatment of Arbacia eggs with the various chlorides employing method 1 e.g. centrifuging the eggs with the electrolytes. The curves represent the average of ten experiments with each salt. The ordinates represent percentage development and the abscissæ the time of immersion in the salt solution before fertilization. It is evident from the chart that the cations can be arranged in the following toxicity series.

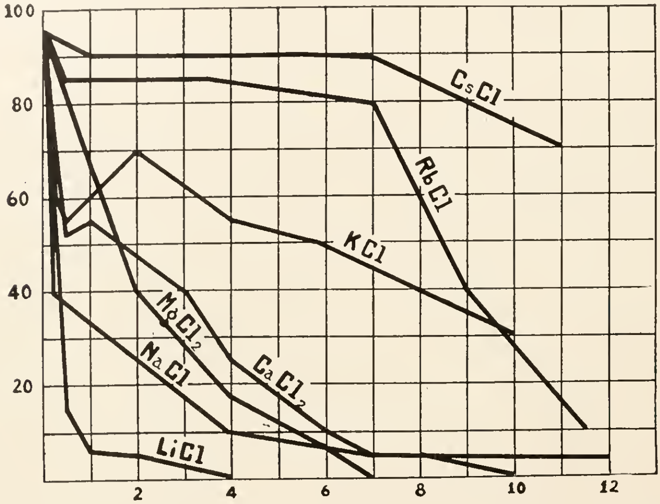

FIG. 1. Toxic effect of pure electrolytes on *Arbacia* eggs. The ordinate represents percentage of development after immersion for the period in hours in the electrolyte as represented by the abscissa. Method 1 in which centrifuging was employed to remove sea water.

Li > Na > Mg, Ca > K > Rb > Cs.

Figures 2 and 3 show graphically the results of the average of nine experiments in which the divisions per egg corrected by the control is plotted against the time in the electrolyte. In Fig. 2 the eggs were allowed to develop for one and one-half hours after insemination and in Fig. 3 three hours, before being fixed in formalin and counted.

Potassium curiously enough exhibits a slight stimulation to division from two to three hours after treatment. Both methods bring this out. $MgCl_2$ seems to be a little less toxic than $CaCl_2$ when centrifuging is not employed. We have the feeling that the toxicity of $CaCl_2$ depends somewhat on the quality of the salt used

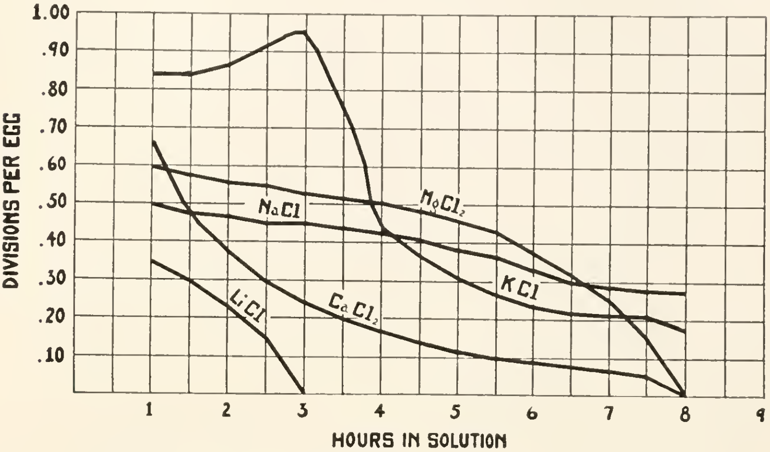

FIG. 2. The toxic effect of pure electrolytes on *Arbacia* eggs. The ordinates represent number of divisions per egg and the abscissæ represent hours in solution. Eggs were allowed to develop 1 ½ hours in sea water after fertilization. In Fig. 2 and Fig. 3 the number of divisions per egg has been corrected by the control.

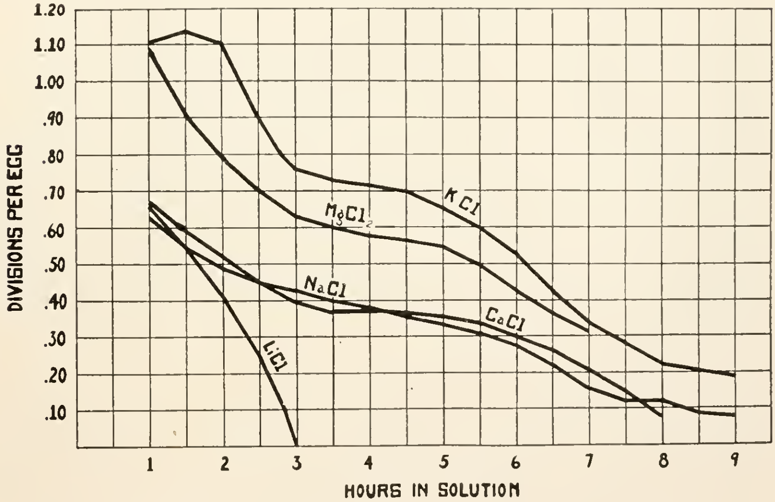

FIG. 3. Same as Fig. 2 except samples counted 3 hours after fertilization.

The least toxic seems to be that of Poulenc (France). LiCl is uniformly the most toxic salt tested.

In order to show the effect of the presence of small amounts of sea water on the toxicity of the salt, eggs centrifuged three times adding fresh electrolyte solution after each centrifugation to rid them of sea water, were placed in varying mixtures of sea water and the electrolyte under investigation.

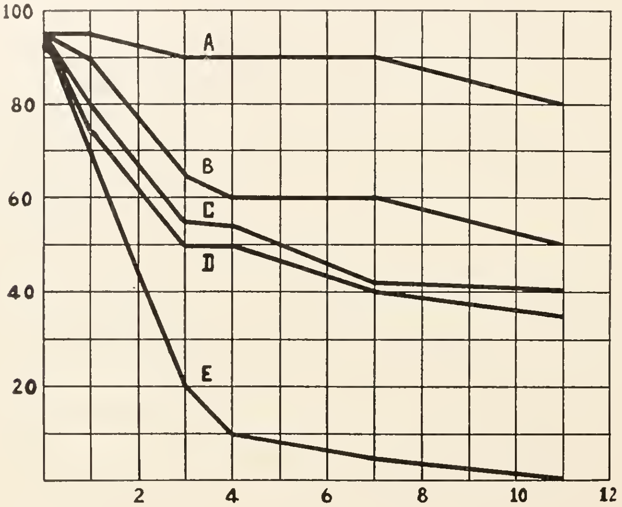

FIG. 4. Effect on toxicity of electrolytes when varying amounts of sea wate1 are present. Ordinates represent percentage development after immersion in the electrolyte solution and abscissæ the numbers of hours of treatment. Curve *A* represents a mixture of 10 parts $CaCl_2$ plus 30 parts sea water; Curve *B* = 22 parts $CaCl_2$ plus 18 parts sea water; Curve C = 28 parts $CaCl_2$ plus 12 parts sea water; curve *D* = eggs centrifuged once from surrounding sea water and put in isotonic $CaCl_2$; curve *E* = eggs centrifuged three times with pure $CaCl_2$ to remove all trace of sea water.

The following figures (4 and 5) illustrate these results with Ca, Na and K. The other salts show similar effects. As in Fig. 1 the ordinates represent percentage of eggs which fertilized developed into the swimming stage and the abscissæ the time of treatment with the electrolyte before fertilization.

The smallest amount of sea water present reduces the salt toxicity in a most impressive manner. It makes one realize what a potent agent ionic antagonism may be.

It seems perfectly clear that the toxicity of the electrolytes as measured by the ability of the egg to be fertilized and subsequently develop, determined by two separate methods and two observers, follows the series Li > Na > Ca, Mg > K > Rb > Cs. What is not clear is the interpretation of these results.

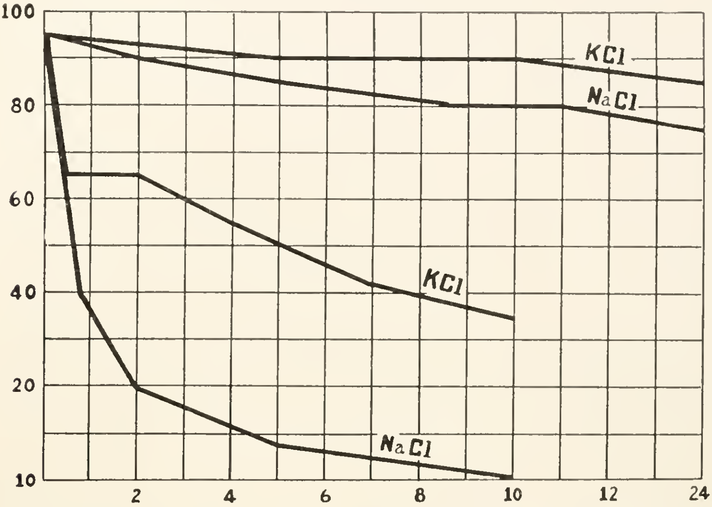

FIG. 5. Effect on toxicity of NaCl and of KCl of varying amounts of sea water Ordinates represent percentage development after immersion for the period in hours represented by the abscissæ. The two upper curves show the toxicity when the eggs are centrifuged once and placed in the isotonic salt. The two lower curves represent the toxicity when the eggs are centrifuged three times with the electrolyte.

It is suggestive that the atomic numbers, show a series very nearly identical with the order of toxicity found in this study. In order of increasing atomic number the series runs Li, Na, Mg, K, Ca, Rb, Cs.

However it is probably fortuitous that the toxicity series (with the exception of K) found for the Sea Urchin egg closely parallels the atomic number series. Any proof to the contrary must consider the action of the salts on at least the more important protein, lipoid, fat and mineral systems which constitute protoplasm and the complex interrelations of these various systems. It is possible however that this parallelism may represent the dominance of the chemical constitution of the reagent in the reaction between protoplasm and the salt.

The problem is further complicated by the fact that Chambers and Reznikoff (35, 36) have shown that the toxicity of the salts for fresh water Amœbæ immersed in the solution is

$$K > Na > Ca > Mg,$$

but when the electrolyte is injected into the interior of the protoplasm quite the reverse relation holds, viz.,

$$Ca > Mg > Na > K.$$

These results seem to implicate the chemically active plasma membrane at the surface of the cell and possibly the difference in type of protoplasm ("interior" and "exterior") as suggested by Chambers (37) and Page, Chambers and Clowes (38).

SUMMARY.

1. The toxicity of the following cations used as chlorides for the Arbacia egg was found to be

$$Li > Na > Ca,\ Mg > K > Rb > Cs.$$

2. It has been suggested that the term "toxicity" is applied to a miscellany of the most heterogeneous reactants in protoplasm hence as an entity its prediction and formulation in our present state of knowledge is not probable.

REFERENCES.

1. **Loeb.** Arch. f. ges Physiol. LXVII., 1 (1897).
2. **Loeb.** Ibid., LXXI., 457 (1898).
3. **Loeb.** Ibid., LXXV., 303 (1899).
4. **Loeb.** Festschrift. für Fich (1899), 101.
5. **Greene.** Am. Jour. Physiol., II., 126 (1898).
6. **Lingle.** Am. Jour. Physiol., IV., 264 (1900).
7. **Loeb.** Am. Jour. Physiol., III., 327 (1900).
8. **Lillie.** Am. Jour. Physiol., V., 56 (1901).
9. **Overton.** Arch. f. ges Physiol., XCII., 346 (1902). Ibid., CV., 176 (1904).
10. **Mathews.** Am. Jour. Physiol., XI., 455 (1904).
11. **Lillie.** Am. Jour. Physiol., XXVI., 106 (1910).
12. **Chambers and Reznikoff.** Jour. Gen. Physiol., VIII., 369 (1926).
13. **Maxwell.** Am. Jour. Physiol., XIII., 154 (1905).
14. **Lillie.** Am. Jour. Physiol., XXIV., 459 (1909).
15. **Loeb.** Jour. Biol. Chem., XXVII., 339, 353, 363 (1916).
16. **Loeb.** Jour. Biol. Chem., XIX., 431 (1914).

17. **Loeb.** Jour Gen. Physiol., III., 237 (1921).
18. **Gellhorn.** Arch. ges Physiol., CC., 583 (1923).
19. **Magowan.** Bot. Gaz., XLV., 45 (1908).
20. **Osterhout.** Bot. Gaz., XLVIII, 98 (1909).
21. **Lipman.** Bot. Gaz., XLVIII., 105 (1909).
22. **Höber.** "Physikalische Chemie der Zelle und der Gewebe," 1924, p. 594.
23. **Furuhata.** Jour. Immunology, III., 423 (1918).
24. **Gros.** Arch. Exp. Path. u. Pharm., LXII., 1.
25. **Wright and MacCallum.** Jour. Path. and Bact., XXV., 316 (1922).
26. **Neuhausen and Breslin.** Johns Hopkins Hosp. Bul., XXXIV., 199 (1923).
27. **Koch.** Jour. Biol. Chem., III., 53 (1907).
28. **Koch and Williams.** Jour. Pharm. Exp. Ther., II., 253 (1910).
29. **Clowes.** Jour. Phys. Chem., XX., 407 (1916).
30. **Neuscholsz.** Flügers Arch., CLXXXI., 17 (1920).
31. **Bancroft.** Jour. Phys. Chem., XIX., 349 (1915).
32. **Walden.** Personal Communication.
32. **Page.** Proc. Soc. Exp. Biol. and Med., XXII., 117 (1924).
33. **Heilbrunn.** Science, LXI., 236 (1925).
34. **Chambers.** Personal Communication.
35. **Chambers and Reznikoff.** Proc. Soc. Exp. Biol. and Med., XXII., 320 (1925).
36. **Reznikoff and Chambers.** Proc. Soc. Biol. and Med., XXII., 386 (1925).
37. **Chambers.** Jour. Gen. Physiol., IV., 41 (1921-22).
38. **Page, Chambers and Clowes.** Jour. Exp. Zoöl., XIL., 235 (1925).

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